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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE **PATENTS**

Practitioner's Docket No.: 1372.143.PRC

In re Application of:

David L. Vesely

Serial No.: 10/708,688 Art Unit: **1653**

Filed: 3/18/2004 Examiner: **Marsha M. Tsay**

For: **CANCER TREATMENT USING PROANP PEPTIDES**

Rule 132 Declaration

Upon being duly cautioned, I depose and state as follows:

1. My name is David L. Vesely, M.D., Ph.D. I am employed by the Department of Internal Medicine and the Department of Physiology and Biophysics at the University of South Florida. I am the Director of the Cardiac Hormone Center at the University of South Florida Health Sciences Center, and I am the Chief of Endocrinology and Metabolism at the J.A. Haley Veterans Administration Medical Center, whose address is 13000 Bruce B. Downs Blvd., Tampa, Florida, 33612. My primary responsibility is patient care. I have been a practicing medical doctor for 33 years and a medical researcher for 35 years. My education and other data are included in my curriculum vitae attached hereto.
2. Subsequent to the filing of the application, referenced above, experiments were conducted by myself to test the effectiveness of the four peptide hormones from the atrial natriuretic peptide prohormone to inhibit the growth of carcinomas from human breast adenocarcinoma cells and from small-cell lung cancer of the lung.
3. A journal article titled Four peptide hormones decrease the number of human breast adenocarcinoma cells was published in the January 2005 edition of the European Journal of Clinical Investigation (vol. 35, pp 60-69, Jan. 2005), a copy of which is attached hereto as EXHIBIT A.
4. The research protocol employed to test the effect of the of the four peptide hormones from the atrial natriuretic peptide prohormone to inhibit the growth of carcinomas from human breast adenocarcinoma cells was substantially the same as that used in the instant application.

- Cells were subcultured for 24 hours and then seeded to cover slips in 24 well plates.
- Cells were submitted to 24 hours of serum deprivation.
- After 24 hours of serum deprivation, media volume was reduced 250µl per well, with or without the respective peptide hormones at their 1-µM concentrations.
- Cells were then incubated for varying amounts of time (24, 48, 72, and 96 hours)

5. The number of breast adenocarcinoma cells after 24 hours without the addition of any of the peptide hormones averaged 75± 3 cells per high-powered field when ten fields of the cover slip were evaluated at x40 along the x-axis.
6. The addition of 1 µm of long acting natriuretic peptide (LANP) decreased the number of adenocarcinoma cells to 52 ± 3 cells; a 31% decrease (P<0.05).
7. Vessel dilator, at 1µM concentrations for 24 hours, yielded a 60% (P<0.001) decrease in the number of adenocarcinoma cells.
8. Kaliuretic peptide, at 1µm concentrations for 24 hours, yielded a 27% (P<0.05) reduction in breast adenocarcinoma cells.
9. The number of breast adenocarcinoma cells in culture decreased 40% (P<0.01) when exposed to atrial natriuretic peptide (1µm) for 24 hours.
10. A journal article titled Four peptide hormones decrease the number of human small-cell lung cancer cells was submitted to *Clinical Cancer Research* in 2004, a copy of which is attached hereto as EXHIBIT B.
11. The research protocol employed to test the effect of the of the four peptide hormones from the atrial natriuretic peptide prohormone to inhibit the growth of carcinomas from human breast adenocarcinoma cells was identical to that used in regard to human breast adenocarcinoma cells (*supra*).
12. The number of small-cell lung carcinoma cells after 24 hours without the addition of any of the peptide hormone averaged 89±9 cells per high-powered field when ten fields of the cover slip were evaluated at x40 along the x-axis.
13. The addition of 1 µm of long acting natriuretic peptide (LANP) decreased the number of adenocarcinoma cells to 68± 6 cells; a 21% decrease (P<0.05).
14. Vessel dilator, at 1µM concentrations for 24 hours, yielded a 63% (P<0.001) decrease in the number of small-cell lung carcinoma cells.

15. Kaliuretic peptide, at 1μm concentrations for 24 hours, yielded a 30% ($P<0.05$) reduction in small-cell lung carcinoma cells.
16. The number of breast adenocarcinoma cells in culture decreased 39% ($P<0.01$) when exposed to atrial natriuretic peptide (1μm) for 24 hours.
17. The aforementioned studies show that the four peptide hormones from the atrial natriuretic peptide prohormone inhibit the growth of carcinomas irrespective of the site of origin, *i.e.*, breast and pancreatic, and one skilled in the art would expect that they would inhibit the growth of other carcinomas as well (*i.e.* prostate, colon, and lung adenocarcinomas).
18. The data included herein would enable one skilled in the art to apply the teachings of the instant application to all types of cancer without undue experimentation as the agents significantly decrease the number of several, different types of cancer cells.
19. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true and further that false statements and the like so made are punishable by fine, imprisonment or both under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application and any patent issued pursuant thereto.

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JAN 18 2005
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CURRICULUM VITAE

David L. Vesely, M.D. Ph.D. F.A.C.P. F.A.C.E.

Professor of Internal Medicine, Physiology & Biophysics
University of South Florida Health Sciences Center
Tampa, Florida

Medical School Address:

Chief of Endocrinology, Diabetes and Metabolism
Tampa V. A. Medical Center
13000 Bruce B. Downs Boulevard
Tampa, Florida 33612
Telephone: Office (813) 972-7624, Home (813) 977-8776
Fax: (813) 972-7623

Academic and Administrative Experience:

Acting Associate Chief of Staff for Research and Development at the James A. Haley Veterans Medical Center and Acting Chief of Endocrinology, Diabetes and Metabolism, University of South Florida Medical School, 2004. In addition to Director, University of South Florida Cardiac Hormone Center (Tampa), and Chief of Endocrinology, Diabetes and Metabolism, James A. Haley Veterans Medical Center, Tampa, Florida.

Director, University of South Florida Cardiac Hormone Center, 1999-present. *University of South Florida, Tampa, Florida.* This is a multidisciplinary center consisting of faculty from Biochemistry and Molecular Biology, Internal Medicine, Obstetrics and Gynecology, Physiology and Biophysics.

Chief, Endocrinology, Diabetes & Metabolism, 1993-present. *James A. Haley Veterans Affairs Medical Center, Tampa, Florida.* Provides leadership and training of endocrinology fellows, medical residents and fourth year medical students at James A. Haley Veterans Hospital which has more ambulatory care visits than any other veterans hospital in the United States (over 970,000 clinical visits per year).

Professor of Medicine, 1989-present. *University of South Florida Health Sciences Center and James A. Haley Veterans Affairs Medical Center, Tampa, Florida.* As Attending Physician at Tampa General Hospital, James A. Haley Veterans Medical Center, and Moffitt Cancer Center Hospital, serve as the endocrinology consult physician and provide comprehensive medical care for fee for service, medicare, and managed care patients including faculty and indigent patients while at the same time

providing teaching instruction for third year medical students, medical housestaff, and endocrinology fellows.

Distinguished Visiting Professor (July-Dec. 1995). *The Christchurch School of Medicine, Christchurch, New Zealand.* A mini sabbatical to determine the molecular species of brain natriuretic peptide prohormone and its metabolically active peptides within the human heart and circulation. This was a collaborative investigation with the Endocinology Research Group in Christchurch consisting of Eric Espiner, M.D., Tim Yandle, Ph.D., A. Mark Richards, M.D., and M. Gary Nicholls, M.D.

Professor of Physiology and Biophysics (In addition to Professor of Medicine), 1990-present. *University of South Florida Health Sciences Center, Tampa, Florida.* Directs the research training of graduate and postgraduate PhD and MD students.

Professor of Medicine, 1983-1989. *University of Arkansas for Medical Sciences and Chief of Endocrinology and Metabolism, John L. McClellan Veterans Hospital Little Rock, Arkansas.* Provided leadership in the training of third year medical students, medical residents, and endocrine fellows while also serving as the primary care physician in treating fee for service patients and patients in the federal health care system.

Senior Fogarty International Scholar and Professor of Medicine, 1984-1985. *Institut National de la Sante et de la Recherche Medicale Faculte de Medicine (Pasteur) Nice, France.* Senior Fogarty International Fellowship from United States' National Institutes of Health was utilized to share new technologies with investigators at the Diabetes Branch of the French National Institutes of Health while at the same time learned new molecular biology techniques from French investigators under the direction of Pierre Freychet, M.D.

Associate Professor of Medicine, Acting Chief, Division of Endocrinology and Metabolism, 1979-1983. *University of Arkansas for Medical Sciences Little Rock, Arkansas.* Developed Endocrinology and Metabolism Division at the University from a one member division with no extramural grant funds into a division with five MD endocrinologists and two PhDs with over one million dollars in extramural grant support.

Assistant Professor of Medicine, Acting Chief, Division of Endocrinology and Metabolsim, 1978-1979. *University of Arkansas for Medical Sciences Little Rock, Arkansas.*

Assistant Professor of Medicine, Division of Endocrinology and Metabolism, 1977-1978. *University of Miami School of Medicine Miami, Florida.*

Instructor in Medicine, Department of Medicine, Division of Endocrinology and Metabolism, 1976-1977. *University of Miami School of Medicine Miami, Florida.*

Education:

Creighton University. Omaha, Nebraska, *Bachelor of Science* Biology 1961-1964.

Creighton University, School of Dentistry, Omaha, Nebraska, 1964-1967.

Doctor of Medicine (MD), University of Arizona School of Medicine, Department of Medicine, Tucson, Arizona, 1968-1972.

Doctor of Philosophy (PhD), University of Arizona, Department of Biological Sciences, Tucson, Arizona, 1968-1972.

National Institutes of Health (N.I.H.) Fellow for combined medical and Ph.D. training, 1968-1972.

Postgraduate Training:

Internship, University of Miami Affiliated Hospitals (Straight Medical), Miami, Florida, 1972-1973.

Residency, University of Miami Affiliated Hospitals (Straight Medical), Miami, Florida, 1973-1974.

Fellowship in Endocrinology and Metabolism, Division of Endocrinology and Metabolism, University of Miami, School of Medicine, Miami, Florida,1974-1976.

Personal:

Born: March 6, 1943

Married: Clo Farrell, 1973 (deceased-cancer, December 2002)

Children: Susanna, 1974; Catherine, 1978; Matthew, 1981; Brian, 1984; Jonathan, 1988.

Honors and Awards:

President of Delta Sigma Delta, international dental fraternity, 1965-1967.

President of Dental School Sodality, 1965-1967.

National Institutes of Health Pre-Doctoral Fellow (Biochemistry and Physiology, University of Arizona, Tucson, Arizona), 1968-1972.

Lange Scholarship Award, 1968-1972.

Mosby Scholarship Award, 1968-1972.

National Institutes of Health Post-Doctoral Fellow (Endocrinology Subspeciality Training and Research Fellowship, University of Miami School of Medicine. Miami, Florida), 1974-1976.

Outstanding Clinical Teacher, University of Miami School of Medicine, 1977.

Honors and Awards (cont.):

Physician Recognition Award from American Medical Association, 1977.

Outstanding Young Men of America Award for 1979 awarded by U.S. National Jaycees.

New York Academy of Science, member, 1979.

Who's Who in the South and Southwest, 1980 to present.

Outstanding Clinical Teacher voted by the 1979-1980 Senior Medical Class, 1980.

Invited participant in Gordon Research Conference on Cyclic Nucleotide Metabolism, 1981.

Senior Fogarty International Fellow (N.I.H.) at Institut National de la Sante et de la Recherche Medicale. Nice, France, 1984-1985.

Inducted into American Men and Women of Science for outstanding contributions to the biological sciences, 1986-1987.

Outstanding Clinical Teacher voted by 1987-1988 Senior Medical Class, 1988.

Distinguished Physicians of America Award, 1990.

Native Son Award, 1990. City of Scribner (Nebraska) Award for outstanding achievements by a person whose early education began in Scribner. The Award plaque and portrait of Dr. Vesely will be permanently displayed in the Civic Auditorium. Dr. Vesely received the American Legion Medal as the Outstanding Scholar-Athlete when he graduated from Scribner Public High School in 1961.

The Best of Who's Who in the Health and Medical Sciences, 1991 to present.

The University of Arizona College of Medicine Alumni Medal. This award for an outstanding alumni was presented at the University of Arizona College of Medicine in 1992.

American Heart Association National Council for Basic Science, 1994-present.

Experimental Biology 94 (Federation Meetings) - featured symposium speaker, 1994.

Fellow, American College of Endocrinology, 1995.

Who's Who in Science and Engineering, 1995 to present.

Who's Who in American Education, 1996 to present.

Honors and Awards (cont.):

Featured speaker at Society of Nephrology, Republic of China, 1996.

United States Patent No. 5,691,310, 1997.

Who's Who in the World, 1998 to present.

Who's Who in Medicine and Health Care, 1998-present.

One Thousand Great Americans – The International Biographical Centre of Cambridge, England, 2001.

Featured Plenary Speaker – On Atrial Natriuretic Peptides in the New Millennium at the European Renal Association Annual Meeting, June 24-27, 2001, Vienna, Austria.

Lifetime Achievement Award – The International Biographical Centre of Cambridge, England, 2001.

Experimental Biology 2002 - Featured Speaker and Chair of Symposium on "Diagnosis and Treatment with Atrial Natriuretic Peptides", April 20-24, 2002, New Orleans, Louisiana.

United States and International Provisional Patent 02 B065 PR, 2003.

America's Top Physicians, Consumer Research Council of America, 2003, 2004, 2005.

United States and International Provisional Patent 04B068 PR, 2004.

Featured Symposium Speaker and Chair of Symposium – "Diagnosis and Treatment Utilizing Natriuretic Peptides". International Physiological Congress/Experimental Biology Meeting 2005. March 31-April 5, 2005, San Diego, California. (20,000+ scientists attending)

University, State and National Service:

University:

Endocrine, Diabetes, and Metabolism Clinics-weekly/twelve months/year (University and Veterans' Administration clinics) (1978 to present).

Medicine Clinic Attending--twice per week throughout the year at University until 1985. From 1985 on, weekly at Veterans Administration Endocrine/Diabetic Clinics to present.

Internal Medicine Ward Attending - 4 months/year (1978-2000). One month/year (2000-).

Physical Diagnosis: Since 1976, yearly has taught physical diagnosis.

Internship Applicant Interviewer.

Research Council for Research Development of the University of Arkansas for Medical Sciences (1978-1981).

Instructional Development and Education Awards Committee (1979-1989).

Research Advisor to Dean's Institutional Self Study Analysis of the College of Medicine for the National Liaison Committee on Medical Education (1979-1989).

Clinical Affairs Advisor to Dean of College of Medicine for Institutional Self Analysis for the National Liaison Committee on Medical Education (1985-1989).

Product Evaluation and Standards Committee, University of Arkansas for Medical Sciences (1982-1989). Chairman, 1984.

Council for Academic Affairs. University of Arkansas for Medical Sciences (1983-1989).

Goals and Objectives of Medical Residency Committee. University of South Florida Medical School (1991- present).

Dean's Committee for Evaluation of Department of Physiology, University of South Florida Health Sciences Center (1993-1994).

Faculty Council, University of South Florida College of Medicine (1993-1997).

Search for Chair, Department of Physiology, University of South Florida Health Sciences Center (1994-1995).

Search Committee for two new faculty members in Department of Physiology, University of South Florida Health Sciences Center (1997).

Search Committee for a new faculty member in Department of Physiology, University of South Florida Health Sciences Center (1998).

University of South Florida Research and Creative Scholarship Program (1994 to present).

Search for Director, Division of Cardiology, Department of Internal Medicine, University of South Florida Health Sciences Center (2000).

Search for Director, Renal Division, Department of Internal Medicine, University of South Florida Health Sciences Center (2000).

Review of Research and Grant Proposals, Department of Medicine, University of South Florida Medical School (2002-present).

Medical School Representative for University of South Florida to Council of Southern Society for Clinical Investigation (2002-present).

State and Federal:

Research and Development Committee, John L. McClellan Veterans Affairs Hospital, Little Rock, Arkansas (1987-1989).

Research and Development Committee, James A. Haley Veterans Hospital, Tampa, Florida (1989-2001).

Research Advisor to Arkansas Chapter of American Diabetes Association (1978-1989).

Chairman, Professional Committee, Arkansas Chapter of American Diabetes Association. (1982-1985).

Made a one-hour videotape "Diabetes and Its Complications," for Arkansas Public Television Network (1981). After being shown locally it was syndicated and has been shown on the National Public Television Network.

Yearly lectures on "Diabetes Mellitus and Its Management" at Area Health Education Center in Fayetteville; at the Veterans' Administration Hospital in Fayetteville, and the Area Health Education Center in Pine Bluff. (1978-1989)

Yearly lectures on various endocrine conditions at Little Rock Air Force Base Hospital. (1978-1989)

Yearly lectures on endocrine diseases - Orlando Regional Medical Center, Orlando, Florida (1989-), Sarasota Regional Medical Center, Sarasota, Florida (1990-), Winter Park Memorial Hospital, Winter Park, Florida (1990-), and Sun Bay - Humana Hospital, St. Petersburg, Florida (1990-present).

Hillsborough County Public Schools and Tampa Catholic High School (Tampa, Florida) Teach In - Presentation to various grade schools and high schools of what a physician does and how new medical discoveries benefit people (1990-present).

High School Science Teachers Summer Institute - 1991-Seminars on newest advances in medical science for teachers in the Hillsborough County School System.

Drug Utilization and Evaluation Committee, James A. Haley Veterans Hospital (Tampa, Florida). This committee determines the utilization pattern and cost-effectiveness of drugs that are to be placed in the hospital pharmacy (1993-present).

American Heart Association, Florida Affiliate, Research Peer Review Committee (1993-1997).

Director's Risk Management Committee, James A. Haley Veterans Affairs Hospital (Tampa, Florida). Department of Medicine representative to committee that evaluates all potential lawsuits for Veterans Administration Hospital and advises the Director

whether standard of care was met and whether potential lawsuit has any merit (1993-present).

Director of Diabetic and Endocrinology Clinics - John L. McCelllan Veterans Medical Center, Little Rock, Ark. - 1985-1989, James A. Haley Veterans Administration Hospital, Tampa, Florida - 1993-present.

Chairman of Diabetes Advisory Committee (to advise Director and Chief of Staff on all matters relating to diabetic patient care), James A. Haley Veterans Administration Hospital, Tampa, Florida, 1993- present.

Made one-hour videotape "Prostate Cancer" for Florida Public Television Network (WEDU) in 1999. After being shown locally it was syndicated for National Public Television Network.

First Annual African-American Men's Health Forum, Tampa, Florida (2/26/2000). Featured speaker on "Diabetes Mellitus in African-American Men".

Second Annual African-American Men's Health Forum, Tampa, Florida (2/24/01). Featured speaker on "Understanding Your Risk for Diabetes".

National:

Reviewer for Am. Heart J., Am. J. Med, Sci., Am. J. Physiology, Archives of Internal Medicine, Biochim et Biophys Acta, Cardiovascular Research, Circulation, Endocrinology, Peptides, Metabolism, Journal of Clinical Endocrinology and Metabolism, and Science.

Lectured on "Endocrine Emergencies" for the American College of Chest Physicians Critical Care Course in St. Louis, MO.

Membership Committee. The Endocrine Society (1983-1986), Chairman (1985-1986).

Department of Veterans Affairs Merit Review Study Section Grant Reviewer (1989-).

National Institutes of Health Study Section: Biochemical Endocrinology. Special Reviewer (1990-).

American Heart Association National Council for Basic Science (1994-present).

American Heart Association - National: Review of investigator originated research grants (1996-).

Editorial Board - The American Journal of the Medical Sciences (2002-present).

Academic Relations Task Force – Combined Committee of American Association of Clinical Endocrinologists and The Endocrine Society (2004-2005).

International Scientists Working in Dr. Vesely's Laboratory:

Dr. Marie Rochat, Ph.D., Professor of Pharmacology, Laboratoire de Pharmacie. U.E.R. des Sciences Pharmaceutiques et Biologiques de Grenoble (France). Dr. Rochat did her research for her Ph.D. (1980) in Dr. Vesely's laboratory.

Quingfeng Kong, M.D., Post-Doctoral Fellow, Cardiology Research. Shanghai Medical School. People's Republic of China (1991-1992).

Hanan Farghaly, M.D., Post-Doctoral Fellow. Alexandria University Medical School, Alexandria, Egypt (1998-1999).

Imran Hassan, M.D., Post-Doctoral Fellow. Punjab Medical College, Faisalabad, Pakistan (1999-2000).

American Board of Internal Medicine Certification:

Board Certified in Internal Medicine

Board Certified in Endocrinology, Diabetes, and Metabolism

State Licensure:

Diplomat, National Board of Medical Examiners, 1973
Florida State Medical License 1974 - present
Arkansas State Medical License 1978 - present

Grants:

1. National Institutes of Health Dental Research Scholarship--a scholarship covering all expenses leading to a Ph.D. degree (tuition, research, supplies, etc.) 1968-1972. The fellowship was utilized at the University of Arizona (Tucson, Arizona).

2. National Institutes of Health Post-Doctoral Fellowship (1974-1976). This scholarship paid salary and all research expenses for two years of endocrine research.

3. National Institutes of Health Biomedical Research Support Grant 5S07RR5350, Principal Investigator (1978-1980), $4,500.

4. Intergovernmental Personnel Act (IPA) Award from the National Center of Toxicological Research, Principal Investigator (1979-1981), $140,000.

5. National Institutes of Health Grant 1 FO6 TW00905-01. Senior Fogarty International Fellowship from John E. Fogarty International Center for Advanced Study in the Health Sciences (1984-1985). This Grant was utilized to learn new research techniques at the Diabetes Branch of the National Institutes of Health of France.

6. Research Advisory Group Grant from the United States Veterans' Administration entitled "B complex vitamins. Receptor through protein phosphorylation", Principal Investigator (1986-1987), $10,000.

7. Dean's Committee Award for Outstanding Research. University of Arkansas for Medical Sciences for research entitled "Prohormone ANF factors: physiologic effects", Principal Investigator (1987-1988), $5,000.

8. University of Arkansas Institutional Grant entitled "Three potential antihypertensives: Prepro atrial natriuretic factors", Principal Investigator (1987-1988), $6,000.

9. Mr. and Mrs. Sam Walton Research Award from the University of Arkansas for Medical Sciences Foundation Fund for research entitled "Determination of whether prohormone atrial natriuretic peptides 1-30, 31-67, and 99-126 play a role in hypertension in the elderly", Principal Investigator (1988-1989), $7,200.

10. Merit Review Award from the Department of Veteran Affairs entitled "Pro atrial natriuretic factors: Circulating levels and physiologic effects", Principal Investigator (1989-1992), $200,600.

11. National Institutes of Health Grant HL44868 entitled "Cardiac peptides in the control of blood volume." Co-investigator (1991-1995), $333,440.

12. American Heart Association, Florida Affiliate Grant entitled "The role of ANF Prohormone in the Control of Blood Volume." Co-investigator (1991-1993), $80,000.

13. Merit Review Award from U.S. Department of Veterans Affairs entitled "Pro atrial natriuretic peptides: Physiologic and therapeutic effects." Principal Investigator (1993-1996), $200,000.

14. American Heart Association, Florida Affiliate Grant entitled "New cardiac peptides - Proatrial natriuretic peptides: Physiologic and therapeutic effects." Principal Investigator (1993-1996), $80,000.

15. American Heart Association, Florida Affiliate Grant entitled "The role of the heart in physiological control of the GI tract". Co-Investigator (1993-1995), $80,000.

16. Merit Review Award from U.S. Department of Veterans Affairs entitled "Pro atrial natriuretic peptides: Physiologic and therapeutic effects". Principal Investigator (1997-2000), $270,885.

17. American Heart Association, Grant-in-Aid entitled "Treatment of congestive heart failure with new atrial natriuretic peptides". Principal Investigator (1997-2000), $110,000.

18. National Institutes of Health Grant entitled "Secretion and renal effects of cardiac peptides". Co-Investigator (1999-2003), $532,150.

19. American Heart Association Grant-in-Aid entitled "Treatment of congestive heart failure with new cardiac hormones". Principal Investigator (2000-2002), $120,000.

20. Merit Review Grant from Department of Veterans Affairs entitled "Role of ANP and its receptors in stomach function and disease". Co-Investigator (2000-2003), $353,800.

21. Darren Manelski Foundation (NY, NY) "Four cardiac hormones effects on the growth of human pancreatic adenocarcinomas in whole animals". Principal Investigator (2003-2004), $48,000.

22. Merit Review Grant from United States Department of Veterans Affairs "Role of ANP and its receptors in stomach function and disease". Co-Investigator (2004-2007), $495,000.

23. Merit Review Grant from United States Department of Veterans Affairs "Adult stem cells in mouse models of neurodegeneration". Co-Investigator (2005-2008), $706,300, pending.

24. Merit Award from United States Department of Veterans' Affairs entitled "Four cardiac hormones: Regulation of normal and abnormal growth". Principal Investigator (2005-2010), $750,000, pending.

25. National Institutes of Health Grant entitled "Novel treatment of cancer with cardiovascular hormones". Principal Investigator (2005-2010), $1,812,500, DK1 RO1 CA115411-01, pending.

26. American Heart Association Grant-in-Aid entitled "Cardiovascular hormones: Treatment of primary malignant heart tumors". Principal Investigator (2005-2007), $120,000, pending.

Professional Societies:

American Association of Clinical Endocrinologists

American College of Endocrinology, Fellow

American College of Physicians, Fellow

American Diabetes Association

American Federation for Clinical Research

American Medical Association

The Endocrine Society

International Endocrine Society

International Society for Chronobiology

New York Academy of Sciences

Society of Experimental Biology and Medicine

Southern Society for Clinical Investigation

Honor Societies:

Sigma Xi. North American Research Honorary

Teaching:

Internal Medicine attending four months/year (1978-2000); one month/year 2000-present.

Endocrinology attending five months for in-hospital consults, 12 months per year in endocrinology and metabolism clinics.

Didactic Lectures on Internal Medicine for Junior Medical Students (a separate course from the teaching given on Internal Medicine attending rounds).

Medicine Grand Rounds and Postgraduate courses, mainly teaching at American College of Physicians postgraduate courses.

Clinical Correlation Course in Pathology for sophomore medical students (University of Arkansas for Medical Sciences).

Physical Diagnosis - a ten-week course for sophomore medical students of five hours/day instruction on history and physical examinations (University of Miami and University of Arkansas for Medical Sciences).

Endocrine Emergencies Course for Critical Care Nurses (University of Arkansas for Medical Sciences).

Dietary management of Diabetes Mellitus for dieticians (University of Arkansas for Medical Sciences and University of South Florida For Health Sciences).

Diabetes Insipidus and Inappropriate ADH Secretion for Anesthesiology Department.

Medical Housestaff Orientation Lectures in Endocrinology and medical housestaff noon-time lecturer in Endocrinology (throughout the year at University of Arkansas for Medical Sciences, at Tampa General Hospital, and James A. Haley Memorial Veterans Medical Center, Tampa, Florida).

Family Practice Grand Rounds on Thyroid Disease, Insulin Dependent Diabetes Mellitus, Cushing's Syndrome, Multiple Endocrine Neoplasia, and Prolactinomas (University of Arkansas for Medical Sciences).

Lectures on Thyroid Cancer for Ears-Nose-Throat (ENT) Surgical Faculty and Housestaff (University of South Florida Health Sciences Center).

Applied Pharmacology for Advanced Practice Health Care Professionals (James A. Haley Veterans Hospital, Tampa, Florida).

Diabetic Nurse Practioner Program - Instruction and Supervision - weekly throughout year - (J.A. Haley Veterans Hospital, Tampa, Florida).

Physiology Course for Freshman Medical Students: yearly lectures on Endocrine Physiology.

~~EJE~~ 028065PRE.

Eu. J. Clinical Investigation
in press, 2004.

Four peptide hormones decrease the number of human breast adenocarcinoma cells

B.A. Vesely, S. Song, J. Sanchez-Ramos, S.R. Fitz, S. M. Solivan, W.R. Gower, Jr., and D.L. Vesely

Departments of Biochemistry and Molecular Biology, Internal Medicine, Neurology, Physiology and Biophysics

University of South Florida Cardiac Hormone Center

and

James A. Haley Veteran's Administration Medical Center

Tampa, Florida

Running head: Four peptides decrease the number of cancer cells

This investigation is dedicated to Clo Vesely, mother of the first author and wife of senior author, who died of breast cancer. We thank Charlene Pennington for excellent secretarial assistance. This work has been supported in part by VA Merit Review Grants, Layton Bio Sciences, Inc., Helen Ellis Research Fund, Darren Manelski Foundation, the Center for Aging and Brain Repair, USF and by the Functional Genomics and Molecular Biology Core facilities at the H. Lee Moffitt Cancer Center and Research Institute.

Please address correspondence to:

David L. Vesely, M.D. Ph.D.
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University of South Florida for Health Sciences
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Email: david.vesely@med.va.gov

ABSTRACT

Background: A family of six hormones, i.e., atrial natriuretic peptide, brain natriuretic peptide, C-natriuretic peptide, long acting natriuretic peptide, vessel dilator, and kaliuretic peptide's main known biologic properties are sodium and water excreting and blood pressure lowering.

Methods and Materials: These six hormones, each at their 1-μM concentrations, were evaluated for their ability to decrease the number and/or proliferation of breast adenocarcinoma cells in culture for 24, 48, 72, and 96 hours.

Results: Within 24 hours, vessel dilator, long acting natriuretic peptide, kaliuretic peptide, atrial natriuretic peptide and 8-bromo-cyclic GMP, a cell permeable analog of their intracellular mediator cyclic GMP (1 μM), decreased the number of breast adenocarcinoma cells 60%, 31%, 27%, 40%, and 31%, respectively. There was not any proliferation in the three days following this decrease in breast adenocarcinoma cell number. These same hormones decreased DNA synthesis 69% to 85% ($P<0.001$). BNP and CNP did not decrease the number of breast adenocarcinoma cells or inhibit their DNA synthesis. Vessel dilator, long acting natriuretic peptide, kaliuretic peptide and 8-bromo-cyclic GMP (each at 1 μM) decreased the number of cells in the S phase of the cell cycle by 62%, 33%, 50%, and 39%, respectively (all $P<0.05$). Natriuretic peptide receptors-A and C were present in the breast adenocarcinoma cells.

Conclusions: Four peptide hormones significantly decrease the number of human breast adenocarcinoma cells within 24 hours and inhibit the proliferation of these cells for at least 96 hours. Their mechanism of doing so involves inhibition of DNA synthesis and decrease in cells in the S phase of the cell cycle mediated in part by cyclic GMP.

Key words: Atrial natriuretic peptides; cancer cell proliferation; DNA synthesis; cyclic GMP; cell cycle progression

INTRODUCTION

Atrial natriuretic peptides (ANPs) consist of a family of peptides that are synthesized by three different genes then stored as three different prohormones [i.e., 126 amino acid (a.a.)[1] atrial natriuretic peptide (ANP), 108 a.a. brain natriuretic peptide (BNP), and 126 a.a. C-type natriuretic peptide (CNP) prohormones] [1-3]. Within the 126 a.a. ANP prohormone are four peptide hormones whose main known biologic properties are blood pressure regulation and maintenance of plasma volume in animals [4-10] and humans [11-13]. These peptide hormones, numbered by their a.a. sequences beginning at the N-terminal end of the ANP prohormone, consist of the first 30 a.a. of the prohormone i.e., long acting natriuretic peptide (LANP), a.a. 31-67 (i.e., vessel dilator), a.a. 79-98 (kalluretic peptide) and a.a. 99-126 (ANP) [3,14]. The BNP and CNP genes, on the other hand, appear to each synthesize only one peptide hormone within their respective prohormones, i.e., BNP and CNP [2,15,16]. Each of these peptide hormones circulates in healthy humans with vessel dilator and LANP concentrations in plasma being 15- to 20-fold higher than ANP, BNP and CNP [17-23].

We have recently found that vessel dilator, long acting natriuretic peptide, kaliuretic peptide and ANP decrease the number of human pancreatic adenocarcinoma cells in culture by 65%, 47%, 37% and 34%, respectively, within 24 hours [24]. This decrease was sustained without any proliferation of the adenocarcinoma cells occurring in the three days following this decrease in number [24]. ANP has also been reported to decrease the number of hepatoblastoma cells in culture [25]. The mechanism of these peptide hormones' decrease in pancreatic adenocarcinoma cell number and antiproliferative effects included a 83% or greater inhibition of DNA synthesis but not

owing to enhanced apoptosis, i.e., programmed cell death [24]. One of the known mediators of these peptide hormones' mechanism(s) of action, i.e., cyclic GMP inhibited DNA synthesis in these adenocarcinoma cells by 51% [24]. When the four peptide hormones synthesized by ANP gene are given subcutaneously via osmotic pumps for one week in athymic mice with human pancreatic adenocarcinomas, these peptide hormones completely stop the growth of these adenocarcinomas [26]. Three of the four peptides decreased the volume of the human pancreatic adenocarcinomas by 49%, 28%, and 11% while at the same time the volume of the adenocarcinomas in the placebo-treated animals increased 20-fold [26].

The present investigation was designed to determine if these four peptide hormones' effects are specific to pancreatic adenocarcinomas or whether they might decrease the number of adenocarcinoma cells from an adenocarcinoma that arose in a different site, i.e., breast. This investigation incorporates the atrial peptides made by all three genes within the heart, i.e., the above four peptide hormones plus BNP and CNP. When the four peptide hormones from the ANP gene were found to cause a significant decrease in the number of breast adenocarcinoma cells, it was investigated whether the mechanism(s) of this decrease in the number of breast cancer cells and the ability of these peptides to inhibit further proliferation of these cancer cells after their decreased number was owing to inhibition of DNA synthesis. It was then determined if they influence cell cycle progression.

MATERIALS AND METHODS

Breast Adenocarcinoma Cells. A cell line (ATCC number CRL-2327) of human breast adenocarcinoma cells was purchased from the American Type Culture Association

(ATCC), Manassas, VA. This breast adenocarcinoma cell line was derived in 1995 from a 49 year old Caucasian female who harbored a homozygous deletion in exon four of the fragile histidine triad (FHIT) gene [27]. The FHIT gene, which spans the fragile site 3B (FRA3B) fragile site at chromosome 3p 14.2, is a candidate tumor suppresser gene in breast cancer [27]. There was a familial history of breast cancer with this cancer being present in the maternal grandmother.

Culture of the Breast Adenocarcinoma Cells. Propagation of these cells was in Roswell Park Memorial Institute (RPMI) 1640 medium with 2 mM/L-glutamine adjusted with addition of 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM of 90% sodium pyruvate and heat-inactivated 10% fetal bovine serum (Sigma Chemical Company, St. Louis, MO) at a temperature of 37°C as recommended by the ATCC. Cells were dispensed into new flasks with subculturing every 6-8 days. The growth medium was changed every three days.

Research Protocol

After the breast adenocarcinoma cells were subcultured for 24 hours they were then seeded to coverslips in 24 well plates (Nunclon™, Roskilde, Denmark) with 1 mL of the above RPMI media. After 24 hours, wells were washed twice with 1 mM phosphate buffered saline to remove the fetal bovine serum. Removal of serum was done to remove all variables (EGF, etc.) present in serum in order that interpretation of any data obtained would be straightforward. After 24 hours of serum deprivation, media volume was reduced to 250 µL per well with or without the respective peptide hormones at their 1-µM concentrations (peptide hormones were 1% of this 250 µL volume). The adenocarcinoma cells were then incubated for various periods of time (24,48,72, and 96

hours). The number of breast adenocarcinoma cells were then counted with a cell counter (Thomas Scientific®, Swedesboro, NJ) evaluating ten fields of the microscope slide at 40 x along the X-axis with an Olympus BH-2 microscope (Atlanta, Georgia). This evaluation was repeated on six separate occasions with the number of adenocarcinoma cells reflecting 60 observations for each of the groups, i.e., 60 observations for controls and 60 observations in each of the six groups with the respective peptide hormones. The peptide hormones used in this investigation were from Phoenix Pharmaceuticals, Inc., Belmont, California.

Determination of DNA Synthesis. To investigate whether these peptide hormones were inhibiting DNA synthesis, bromodeoxyuridine (BrdU) incorporation into the adenocarcinoma cells was utilized. BrdU was from BD Bioscience, San Jose, California. DNA synthesis and doubling of the genome take place during the synthetic or S phase [28,29]. BrdU is a thymidine analog incorporated into nuclear DNA during the S phase of the cell cycle [28-31]. After 24 hours in culture with 1 μM of LANP, vessel dilator, kaliuretic peptide, ANP, BNP, or CNP, respectively, or with no peptide hormone (i.e., control), BrdU in a final concentration of 10 μM in the cell culture medium was added for 45 minutes - which is the time in which the cells are in the logarithmic phase of cell proliferation. For immunohistochemistry, a BrdU *in situ* detection kit from Becton Dickinson Immunocytochemistry Systems, San Jose, California, was utilized.

The incorporation of the BrdU stain into the nucleus was counted using a Nikon Inverted Diaphot-TMD Microscope (Tokyo, Japan). The number of stained nuclei were compared in the six peptide hormone groups to the positive control group. The negative control for these studies was provided by Becton Dickinson Immunocytochemistry

Systems (San Jose, California). To investigate DNA synthesis, BrdU incorporation by immunochemistry has been demonstrated to be equally good as ^{3}H-thymidine incorporation [30,31] and has the advantage that it provides high resolution [28,29].

Cyclic GMP Effects on DNA Synthesis. Cyclic GMP is one of the known mediators of the previously described biologic effects of these peptide hormones [4,32,33]. All four of these peptides synthesized by the ANP gene-induced vasodilations of vasculature are mediated by increased cyclic GMP concentrations via enhancing guanylate cyclase activity [4,32,33]. We have previously shown that each of these peptide hormones increase cyclic GMP while simultaneously dilating vasculature [4]. 8-bromo-cyclic GMP reproduces these vasodilatory effects [32]. For the present investigation of the mechanism of these peptide hormones' ability to inhibit DNA synthesis in breast adenocarcinoma cells, 8-bromoguanosine 3',5'-cyclic monophosphate (i.e., 8-bromo-cyclic GMP, Sigma Chemical Co., St. Louis, Missouri) was utilized. 8-bromo-cyclic GMP is a cell-permeable analog of cyclic GMP.

Cell Cycle Analysis. Cell cycle analysis was performed by Becton Dickinson flow cytometer FACScan using Mod Fit LP software (Becton Dickinson, Mountain View, CA) after the respective peptide hormones and cyclic GMP (each at 1 μM) were incubated at 37°C for 48 hours with the human breast adenocarcinoma cells ($1x10^{6}$ cells in each group). The RPMI 1640 media with addition of fresh peptide hormones and/or cyclic GMP was exchanged after 24 hours of incubation of the breast adenocarcinoma cells. After the 48 hour incubation, the adenocarcinoma cells were harvested and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and ice-cold 70% ethanol added for 30 minutes at 4°C to fix the cells. These cells were then centrifuged at 2000

rpm for 5 minutes and the supernatant discarded to remove fixative. These cells were resuspended in 1 ml of phosphate buffered saline (PBS, Sigma Chemical Co., St. Louis, Missouri). The adenocarcinoma cells were then recentrifuged at 2000 rpm for 5 minutes. This step was repeated twice more to remove the ethanol before the addition of 100 µg/ml of ribonuclease (DNAase free, Sigma Chemical Co., St. Louis, Missouri). Cells were lyzed using Triton-X-100. Then 50 µg/ml of propidium iodide (in PBS) was added and incubated overnight (12 hours) prior to analysis by flow cytometry. All flow cytometry studies were performed on 12,000 to 14,000 diploid adenocarcinoma cells counted by the flow cytometer.

ANP Receptors in Breast Adenocarcinoma Cells. When it was found that these ANPs decreased the number of human breast adenocarcinoma cells, it was then evaluated whether breast adenocarcinomas have ANP (natriuretic peptide receptors; NPR) receptors to mediate these effects since it has never previously been investigated whether breast adenocarcinomas have ANP receptors. Western blots of the NPR A- and C-receptors were performed as follows:

Western Blotting. Fifty micrograms of protein extract from breast adenocarcinoma cells, measured by using the bicinchoninic acid (BCA) protein assay kit (Pierce; Rockford, IL), was loaded onto each lane of a discontinuous 3.5/7.5% SDS-PAGE gel (Bio-Rad; Hercules, CA), separated by electrophoresis, and then transblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) for 85 min at 0.5 A in Towbin buffer. Blots were blocked for 1 hour at room temperature in a 5% solution of dry milk and then incubated for 1 hour in a 5% solution of bovine serum albumin (Fraction V; Fischer Scientific, Fair Lawn, NJ) in Tris-buffered saline that

contained a 1:2,500 dilution of A035 polyclonal antibody directed against the COOH terminus of the NPR-A receptor protein (generously provided by Dr. David L. Garbers, University of Texas Southwestern, Dallas, TX) or containing Tris buffered saline with a 1:400 dilution antibody to the NPR-C receptor (kindly provided by K. Omori, Osaka, Japan). After being washed with Tris-buffered saline, the membranes were incubated for 1 hour at room temperature in a solution of dry milk with a 1:15,000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG antibody (Amersham Life Sciences, Arlington Heights, IL). After a second washing with Tris-buffered saline, the bands were identified by enhanced chemiluminescence reagents (ECL Plus Kit; Amersham Pharmacia Biotech, Arlington Heights, Illinois) and visualized in a luminescent image analyzer (model LAS-1000; Fujifilm, Tokyo, Japan). Specificity was revealed by the presence of a signal in rat lung (positive control) and absence of a signal with normal rabbit serum, rabbit IgG, and after preabsorption of the NPR-A antibody with NPR-A protein or preabsorption of the NPR-C antibody with NPR-C protein.

RESULTS

Decrease in Number of Breast Adenocarcinoma Cells by Four Peptide Hormones Synthesized by the ANP Gene. The number of breast adenocarcinoma cells after 24 hours without the addition of any of the peptide hormones averaged 75 ± 3 cells per high-powered field when ten fields of the coverslip were evaluated at x 40 along the *x*-axis with an Olympus BH-2 microscope. This evaluation was repeated on six separate occasions with the above number reflecting sixty observations of the number of control adenocarcinoma cells and sixty observations of each of the six

groups with addition of one of the cardiac hormones (Fig. 1). The addition of 1 μM of LANP for 24 hours decreased the number of adenocarcinoma cells to 52 ± 3 cells i.e., a 31% decrease ($P<0.05$) in the number of adenocarcinoma cells with the LANP (Fig. 1). Vessel dilator at 1 μM for 24 hours had an even more dramatic decrease (60%; $P<0.001$) in the number of these adenocarcinoma cells (Figure 1). Vessel dilator decreased the number of cancer cells from 75 ± 3 cells to 30 ± 2 breast adenocarcinoma cells. Kaliuretic peptide at 1 μM for 24 hours decreased the number of breast adenocarcinoma cells 27% ($P<0.05$), i.e., to 55 ± 3 adenocarcinoma cells (Fig. 1).

The number of breast adenocarcinoma cells in culture decreased 40% ($P<0.01$) when exposed to atrial natriuretic peptide (1 μM) for 24 hours (Fig. 1). BNP and CNP, each at 1 μM, only decreased the number of breast adenocarcinoma cells 0% and 2%, respectively, after 24 hours of incubation (not significant). Thus, with respect to their ability to inhibit the growth of adenocarcinoma cells when these cells were exposed to identical concentrations of these six peptide hormones for 24 hours, vessel dilator > ANP > LANP > kaliuretic peptide > CNP > BNP. When the number of cells was examined immediately after the incubation of the respective peptide hormones within the wells, there was not any decrease in the number of cancer cells. In the wells with decreased number of wells secondary to the cardiac hormones, there was evidence of cellular debris.

Decreased Cellular Proliferation After Initial Decrease In Adenocarcinoma Cell Number. When these breast adenocarcinoma cells were exposed for longer periods of time e.g., 48, 72, and 96 hours to vessel dilator, LANP, kaliuretic peptide,

ANP, BNP, and CNP each at 1 μM, there was a nearly complete inhibition of proliferation of the breast adenocarcinoma cells after the decrease in the number of these cancer cells at 24 hours by the peptide hormones from the ANP gene (Fig. 2). Thus, when exposed to vessel dilator, LANP, kaliuretic peptide and ANP for 48 hours the inhibition of the number of cancer cells compared to untreated breast cancer cells was 60% ($P<0.001$), 34%, 34% and 38% ($P<0.05$ for these three peptides), respectively. At 72 hours and 96 hours, the decrease in number of breast adenocarcinoma cells secondary to vessel dilator was 58% and 51% ($P<0.001$, Fig. 2). LANP for 72 and 96 hours resulted in the number of adenocarcinoma cells being reduced 39% and 28% ($P<0.05$ for both) compared to untreated adenocarcinoma cells at these time periods. At both 72 and 96 hours, the number of cancer cells with kaliuretic peptide present was decreased by 39% compared to untreated adenocarcinoma cells ($P<0.05$ for each) (Fig. 2). The number of adenocarcinoma cells at 72 and 96 hours decreased secondary to ANP 38% and 32% ($P<0.05$ for both) compared to the number of adenocarcinoma cells at these same time periods without the addition of any peptide hormone (Fig. 2). Thus, proliferation was inhibited by these cardiac peptide hormones for three days after the initial decrease in cell number in the first 24 hours (Fig. 2). There was not any increase in proliferation of any of the cancer cells when exposed to these four peptide hormones for 1, 2, and 3 days after the initial decrease in number of the breast adenocarcinoma cells within the first 24 hours. There was no significant decrease in breast adenocarcinoma number secondary to BNP or CNP at 48, 72, or 96 hours (Fig. 2).

Cyclic GMP Decreases Breast Adenocarcinoma Cell Number. Cyclic GMP at 1 μM decreased the number of human breast adenocarcinoma cells 31% at 24 hours (Fig. 1). The decrease in number of human breast adenocarcinoma cells at 48, 72, and 96 hours was 36%, 34%, and 36%, respectively ($P<0.05$ at all time points compared to control) (Fig. 2).

Inhibition of DNA Synthesis by Four Peptide Hormones. To help determine the mechanism of the adenocarcinoma cells' decrease in number and decreased cellular proliferation by the four peptide hormones which decreased the number of breast adenocarcinoma cells, we next investigated whether their effects were due to an inhibition of DNA synthesis as they have been demonstrated to decrease DNA synthesis in pancreatic adenocarcinoma cells [24]. Vessel dilator, LANP, kaliuretic peptide and ANP each at their 1 μM concentrations inhibited DNA synthesis when incubated with adenocarcinoma cells for 24 hours by 85%, 70%, 69% and 71%, respectively ($P<0.001$ for each) (Fig. 3). There was no significant decrease in DNA synthesis in the breast adenocarcinoma cells secondary to BNP or CNP (Fig. 3).

Cyclic GMP Inhibits DNA Synthesis in Adenocarcinoma Cells. To help define the mechanism(s) for these peptide hormone's ability to decrease DNA synthesis, one of the known mediators of these peptides biologic effects, i.e., cyclic GMP [4,32,33] was investigated to determine if it could inhibit DNA synthesis in these same breast adenocarcinoma cells. 8-bromo cyclic GMP decreased DNA synthesis in breast adenocarcinoma cells by 54% ($P<0.01$) at its 1 μM concentration (Fig. 3).

Cell Cycle Arrest of Adenocarcinoma Cells With Natriuretic Peptides. Cell cycle progression was directly affected by several of the natriuretic peptides. The majority of

the natriuretic peptides had their strongest modification of cell cycle progression in the S phase of the cell cycle. Vessel dilator decreased the percent of cells in S phase by 62% ($P<0.01$) while kaliuretic peptide decreased the percent of cells in S phase by 50% ($P<0.01$) (Table 1). LANP and 8-bromo cGMP decreased the percent of cells in S phase by 33% and 39% respectively (both significant at $P<0.05$) (Table 1). ANP and CNP decreased percent of cells in the S phase but their decrease did not reach statistical significance. BNP had no effect on the S phase or any other portion of the cell cycle (Table 1). ANP caused a 40% decrease in the G_2-M proliferative phase ($P<0.05$). ANP was the only natriuretic peptide to decrease the percent of cells in the G_2-M phase of the cell cycle. There was an accumulation of cells in the G_0-G_1-phase secondary to vessel dilator, LANP, kaliuretic peptide and ANP (all at $P<0.05$; Table 1). Vessel dilator, which caused the largest decrease in cells in the S phase, had the largest accumulation of cells in the G_0-G_1-phase (Table 1). None of the natriuretic peptide affected the G_2-G_1 phase of the cell cycle. Each of the peptide hormones except BNP caused an increase in cellular debris when examined by flow cytometry (Table 1 and Fig. 4). In figure 4, where control, 8-bromo-cyclic GMP and ANPs' treated breast adenocarcinoma cells evaluation by flow cytometry are illustrated, one observes that S phase of the cell cycle, i.e., the second peak, was decreased in the LANP, vessel dilator, kaliuretic peptide, and 8-bromo-cyclic GMP-treated cells compared to the control flow cytometry curve. There was not a decreased second peak in the BNP treated cells. One also observes in Fig. 4 that G_2-M peak (i.e., third peak) was decreased in the ANP treated cells compared to the control and all of the other natriuretic peptide treated breast cancer cells.

NPR A- and C-Receptors are Present in Breast Adenocarcinoma Cells. It has never previously been investigated whether breast adenocarcinomas have NPR-A and -C receptors. When the breast adenocarcinoma cells were evaluated by Western blots, natriuretic peptide receptors (NPR)-A and C were demonstrated to be present (Fig. 5).

DISCUSSION

This investigation is the first evidence that vessel dilator, ANP, LANP and kaliuretic peptide can decrease the number of breast adenocarcinoma cells. All four of these peptide hormones have been investigated for their effects on only one cancer previously, i.e., human pancreatic adenocarcinomas. Similar results were found with human pancreatic adenocarcinomas with each of the four peptide hormones synthesized by the ANP gene decreasing the number of human pancreatic adenocarcinoma cell during the first 24 hours 34% to 65% [24]. Thus, these peptide hormones significantly decrease the number of adenocarcinoma cells whether breast or pancreas is the original site of their development. The present investigation indicates, therefore, that these peptide hormones are not specific for pancreatic adenocarcinomas and suggests that since they decrease the number of adenocarcinoma cells from two different sites that they may decrease the number of adenocarcinoma cells irrespective of the site of origin. The ability of these peptide hormones to decrease the number of adenocarcinoma cells may have implication(s) for adenocarcinomas at other sites in the body with the majority of cancers of the lung, colon and prostate also being adenocarcinomas [34,35].

This is the first investigation of BNP and CNP on any cancer cell and neither of these peptide hormones had any significant effect on the number of the breast

adenocarcinoma cells. Thus, the effects on breast adenocarcinoma cells appear specific for the peptide hormones synthesized by the atrial natriuretic peptide gene as the peptides synthesized by the BNP and CNP genes had no significant effect on these cancer cells.

Vessel dilator was the most potent of these peptide hormones in decreasing the number of the breast adenocarcinoma cells. Vessel dilator had significant ($P<0.001$) effects within 24 hours (60% decrease in number of adenocarcinoma cells) and inhibited any further proliferation of the adenocarcinoma cells from 24 to 96 hours ($P<0.001$). Vessel dilator also decreased human pancreatic adenocarcinomas *in vitro* the most [24] and decreased pancreatic adenocarcinoma tumor volume the most *in vivo* [26] suggesting that it has the most significant anticancer properties of these peptide hormones.

The other three peptide hormones synthesized by the ANP gene effects on decreasing the number of breast adenocarcinoma cells was significant, however. Atrial natnuretic peptide decreased the number of breast adenocarcinoma cells at 24 hours (40% decrease), which is more than its ability to decrease the number of pancreatic adenocarcinoma cells at 24 hours, i.e., 34% [24]. Kaliuretic peptide, on the other hand, ability to decrease the number of adenocarcinoma cells at 24 hours was not as good with breast adenocarcinoma cells (27%) as with pancreatic adenocarcinoma cells (37%). Thus, there appears to be a difference in these peptide hormones' ability to decrease adenocarcinoma cell number depending upon the site of origin of the adenocarcinoma. In the breast adenocarcinomas, kaliuretic peptide ability to decrease the number of adenocarcinoma cells increased with time with a 39% decrease at 72 and

96 hours compared to its ability to decrease the number of cancer cells at 24 hours (27%; $P<0.05$ at 72 and 92 hours compared to 24 hours). This delayed killing ability was not seen with the other peptide hormones in the present investigation. It should be noted that there was no decrease in the number of cells when examined immediately after addition of the respective peptide hormones indicating that the data obtained was not due to artifact. It is also important to note that cellular debris was present at 24 hours in the peptide hormone-treated breast cancer cell cultures suggesting that cellular necrosis was occurring. This was further confirmed in the cell cycle flow cytometry studies where it was noted that cellular debris increased with cyclic GMP and each of the natriuretic peptides except BNP (Table 1).

Each of these peptide hormones inhibited 69% to 85% of the amount of DNA synthesis in these breast adenocarcinoma cells. These findings suggest that one important mechanism of action of these peptide hormones to inhibit cancer cell number and their proliferation is via their ability to inhibit DNA synthesis. This finding is similar to the ability of these peptide hormones to inhibit DNA synthesis in pancreatic adenocarcinoma cells [24], but considerably more significant than ANP's ability to inhibit DNA synthesis in hepatoblastoma cells (22%) [25].

With respect to the mechanism of how these peptide hormones inhibit DNA synthesis, one of the second messengers of their biologic effects, i.e., cyclic GMP [4,32,33] was found using 8-bromo cyclic GMP to inhibit DNA synthesis up to 54% in the breast adenocarcinoma cells. Cyclic GMP affected cell cycle progression in the same phase of the cell cycle where the natriuretic peptides had their major effect, i.e., the S or synthetic phase of the cell cycle. 8-bromo cyclic GMP mimicking the effects of

these peptide hormones on DNA synthesis in the same cells suggests that cyclic GMP is one of the mediators of these peptide hormones' ability to inhibit DNA synthesis in breast adenocarcinoma cells. The concentrations of cyclic GMP that inhibited DNA synthesis in the adenocarcinoma cells are identical to the concentrations of cyclic GMP measured within tissues secondary to these peptide hormones [4]. Further evidence that the cyclic GMP inhibition of DNA synthesis in breast adenocarcinoma cells may be important for these peptide hormones' anticancer growth effects is that when cyclic GMP was infused subcutaneously for a week in athymic mice with human pancreatic adenocarcinomas, it inhibited 95% of the growth of these adenocarcinomas compared to placebo-treated adenocarcinomas [26].

To determine where in the cell cycle these peptide hormones might be effecting DNA synthesis, analysis of the cell cycle progression was examined by flow cytometry. Vessel dilator, LANP, kaliuretic peptide, and cGMP significantly decreased the number of cells in the S phase (33 to 62%) with a concurrent accumulation of cells in the G_0-G_1-phase of the cell cycle. Thus, these peptide hormones and cGMP's main effects on DNA synthesis are inhibiting the synthetic S phase of the cell cycle [period of DNA replication] of adenocarcinomna cells with an increase of cells in the resting (G_0-G_1)-phase. ANP was the only natriuretic peptide to decrease the percent of cells in the G_2-M (mitotic) phase of the cell cycle which may account in part for its ability to decrease the number of human breast adenocarcinoma while peptides with similar structures (i.e., BNP and CNP) do not decrease the number of adenocarcinoma cells. Also contributing to the specificity of ANP versus BNP and CNP is that the presence of NPR-A receptor was demonstrated in breast cancer cells for the first time in the present investigation.

ANP binds to this receptor with a stronger affinity than BNP or CNP [14-16] and this knowledge also helps to explain why ANP but not BNP or CNP decrease the number of adenocarcinoma cells. Since BNP and CNP have less affinity for this receptor they need to be present in a higher concentration than ANP to bind to receptor and have biologic effects. BNP and CNP's lack of biologic effects in these cancer cells with the knowledge that the NPR-A receptor is present in these breast cancer cells suggests that they are not activating this receptor in the adenocarcinoma while ANP is binding to and activating this receptor.

None of the peptide hormones had any effect on the G_2-G_1-phase of the cell cycle. The data of the present investigation, which for the first time evaluated if these peptide hormones affect cell cycle progression suggests that vessel dilator, LANP, kaliuretic peptide and ANP may be cell cycle regulatory molecules. Likewise these peptide hormones' intracellular mediator cGMP appears to be a cell cycle regulatory molecule in adenocarcinoma cells.

It is important to note as above that ANP receptors were present in the human breast adenocarcinoma cells to help mediate ANP's effects. Both the A or active ANP (NPR)-receptor and the C or clearance-receptor were present in the breast adenocarcinoma cells. This demonstration of NPR receptors in breast adenocarcinoma cells helps to explain ANP's mechanism of action of decreasing breast adenocarcinoma cells. To have biologic effects, ANP has to bind to its active A-receptor, which has guanylate cyclase within the receptor [14]. Activation of guanylate cyclase increases the intracellular mediator cyclic GMP, which was shown in the present investigation to decrease cell cycle progression itself and to decrease breast adenocarcinoma cell

number within 24 hours. It is of interest that the breast adenocarcinoma cells have developed an NPR-C receptor to clear ANP out of the cell and, thus, decrease ANP's effects. It will be of interest in the future to evaluate aggressive breast adenocarcinomas versus less aggressive breast adenocarcinoma to determine if the more aggressive adenocarcinomas may lack the NPR-A receptor similar to finding that the more aggressive breast adenocarcinomas often lack estrogen and/or progesterone receptors [36].

Although normal human breast tissue has not been examined for the presence of atrial natriuretic peptide receptors, examination of rat mammary gland has revealed ANP receptors in the secreting tissue and larger blood vessels of the rat mammary gland [37,38]. Unlabeled CNP in this model displaced 30% [^{125}I]r ANP binding suggesting that NPR-B receptor may be present in rat mammary gland [38]. Since CNP had no effect in the present investigation and CNP preferentially binds to the NPR-B receptor, this would suggest that the NPR-B receptor was not present in the human breast adenocarcinoma cells of the present investigation.

Breast cancer is the leading cause of death in women in the United States and Western Europe [36]. In one of every nine women born in the United States, breast cancer will develop during their lifetime [36]. On the basis of the concept that breast cancer is frequently already a systemic disease at the time of diagnosis in many patients with stage I and, especially stage II disease having micrometastasis at time of diagnosis, the current standard of care is the early administration of systemic therapy (i.e., adjunct chemotherapy) for breast cancer [36]. There has been an increased risk of leukemia with some of these chemotherapeutic regimens [36]. The data of the present

investigation that these peptide hormones decrease the number of breast adenocarcinoma cells suggests that these peptide hormones, either used alone or in combination might be a good addition to the therapeutic regimen as an adjunct therapy for primary breast cancer since they do not cause leukemia or any secondary tumors. These peptide hormones which circulate normally in the human body [18-23] have no known cytoxic effects to normal cells [24] and only one known side effect [4-16]. This side effect, i.e., hypotension, has only been observed with ANP and BNP and never with vessel dilator, LANP, or kaliuretic peptide [4-16]. These last three peptide hormones with the best safety profile also decrease adenocarcinoma cells *in vitro* the most (24 and present investigation) and decrease adenocarcinoma tumor volume *in vivo* the most of the ANPs [26].

In addition to present adjunct chemotherapy for breast adenocarcinomas causing a small incidence of leukemia, presently utilized chemotherapy very commonly causes toxicity in the form of nausea, vomiting, alopecia, and myelosuppression [36]. Permanent ovarian dysfunction is an important long-term side effect that occurs in many premenopausal patients with present breast cancer chemotherapy treatment [36]. None of these toxicities occur with the respective ANPs [11-14]. The data of the present investigation plus the knowledge that these peptide hormones lack the above side effects suggests that they may be useful as adjunct chemotherapy for primary breast cancer.

When breast cancer becomes metastatic, systemic therapy is the current state-of-the-art therapy [36]. The first general principle in the management of patients with disseminated breast cancer is that cure is not possible with current treatment

modalities, so optimal palliation with the least toxicity is the primary therapeutic goal [36]. Since the peptide hormones that were demonstrated to have anticancer effects for breast cancer in the present investigation have less toxicity than all of the currently utilized anticancer chemotherapeutic agents as outlined above, using one or more of these peptide hormones as an adjunct to currently utilized anticancer agents in order to decrease their toxicity via using lower concentrations of current chemotherapeutics may be a reasonable addition to the treatment of disseminated breast cancer.

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FIGURE LEGENDS

Fig. 1. Decrease in human breast adenocarcinoma cells after 24 hour exposure to 1 μM of long acting natriuretic peptide (LANP), vessel dilator, kaliuretic peptide, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). The decrease in breast cancer cells was significant at $P<0.001$ with vessel dilator, $P<0.01$ with ANP, and a $P<0.05$ with kaliuretic peptide and LANP when evaluated by repeated analysis of variance (ANOVA). There was no significant decrease in breast adenocarcinoma cell number secondary to BNP or CNP when evaluated by ANOVA. The decrease in number of breast adenocarcinoma cells secondary to vessel dilator was significantly greater ($P<0.05$) than the decrease secondary to any of the other natriuretic peptides when evaluated by ANOVA. 8-bromo-cyclic GMP induced decrease in breast adenocarcinoma cells was significant at $P<0.05$ when evaluated by ANOVA. (n=60 for each group)

Fig. 2. Time course of decrease in human breast adenocarcinoma cell number with 1 μM of vessel dilator (□), long acting natriuretic peptide (○, LANP), kaliuretic peptide (▲),atrial natriuretic peptide (ANP, Δ), brain natriuretic peptide (BNP, ●), C-type natriuretic peptide (■), respectively, and cyclic GMP (cGMP, 1 μM, ◆) compared to placebo-treated (◇) adenocarcinoma cells. The decrease in cancer cell number by ANP, LANP, kaliuretic peptide, and cGMP were significant at $P<0.05$ while the decrease secondary to vessel dilator was significant at $P<0.001$ at each time-point compared to placebo when evaluated by repeated analysis of variance (ANOVA). There was no significant decrease in breast adenocarcinoma cell number with either BNP or CNP when evaluated by repeated ANOVA. (n=60 for each point in each group)

Fig. 3. Inhibition of DNA synthesis by vessel dilator, long acting natriuretic peptide (LANP), kaliuretic peptide, and atrial natriuretic peptide (ANP) in human breast adenocarcinoma cells. This inhibition of DNA synthesis is illustrated as the percent of DNA synthesis occurring with the respective peptide hormones, each at 1 μM, versus the amount of DNA synthesis without the addition of any of these peptide hormones. The amount of inhibition of DNA synthesis by each of these peptide hormones was significant at $P<0.001$ when evaluated by repeated analysis of variance (ANOVA). 8-bromo cyclic GMP at its 1 μM concentration inhibited DNA synthesis 54% in breast adenocarcinoma cells ($P<0.01$). BNP and CNP, each at 1 μM, had no significant effect on DNA synthesis when evaluated by repeated ANOVA. Each of the DNA synthesis experiments were performed in duplicate.

Fig. 4. Cell cycle analysis of human breast carcinoma cells under control conditions (A), after treatment with long acting natriuretic peptide (LANP; B), vessel dilator (C), kaliuretic peptide (D), atrial natriuretic peptide (ANP; E), brain natriuretic peptide (BNP; F), C-natriuretic peptide (CNP; G), and cyclic GMP (H), each at 1 μM for 48 hours. The first and largest peak (red) is G_0-G_1 phase of cell cycle. Second peak (blue vertical lines) = S phase. Third peak (red) is G_2-M phase of cell cycle. As one observes, vessel dilator, LANP, kaliuretic peptide, and cyclic GMP had their strongest modification of cell cycle progression in the S phase of the cell cycle. In this flow cytometry evaluation, the area of the curve immediately preceding the G_0-G_1 peak reflects cellular debris. This cell cycle analysis was confirmed with the experiments done several weeks apart.

Fig. 5. NPR A- and C-receptors on human breast adenocarcinoma cells. Western blot analysis with 1:2500 dilution of A035 polyclonal antibody directed against the COOH

terminus of the natriuretic peptide A-receptor (NPR-A) and 1:4000 dilution of antibody to the NPR-C receptor. The upper graph demonstrates the NPR-A receptor in human breast adenocarcinoma (HCC) cells and the positive rat lung control (PANC). The lower graph demonstrates the NPR-C receptor at 66 kilo Daltons (kDa) in the human breast adenocarcinoma cells (HCC) and in positive rat lung (PANC) control. (These Western blots are illustrative of the Western blots, which were performed three times with the same results.)

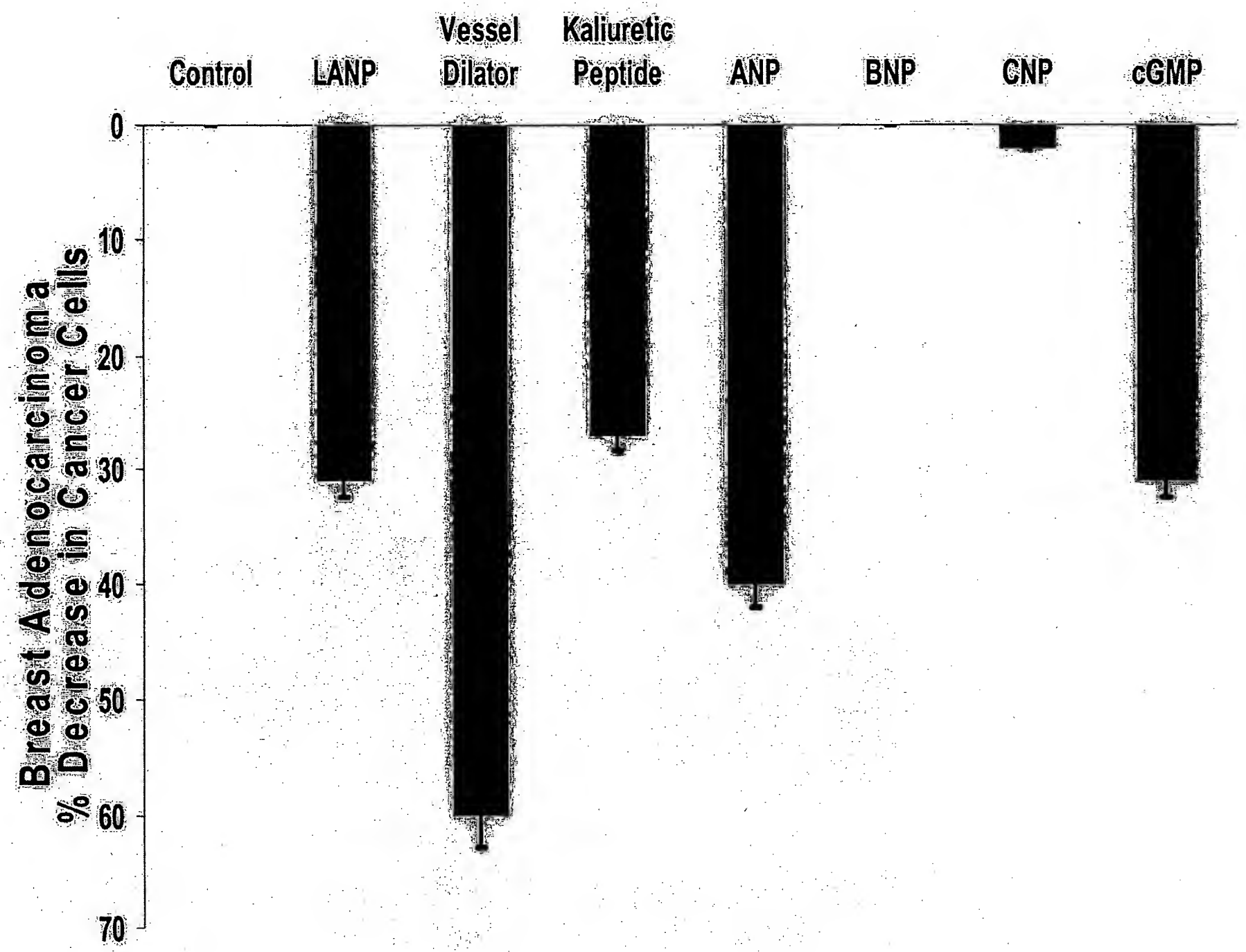
Table 1. Cell cycle analysis of breast cancer.

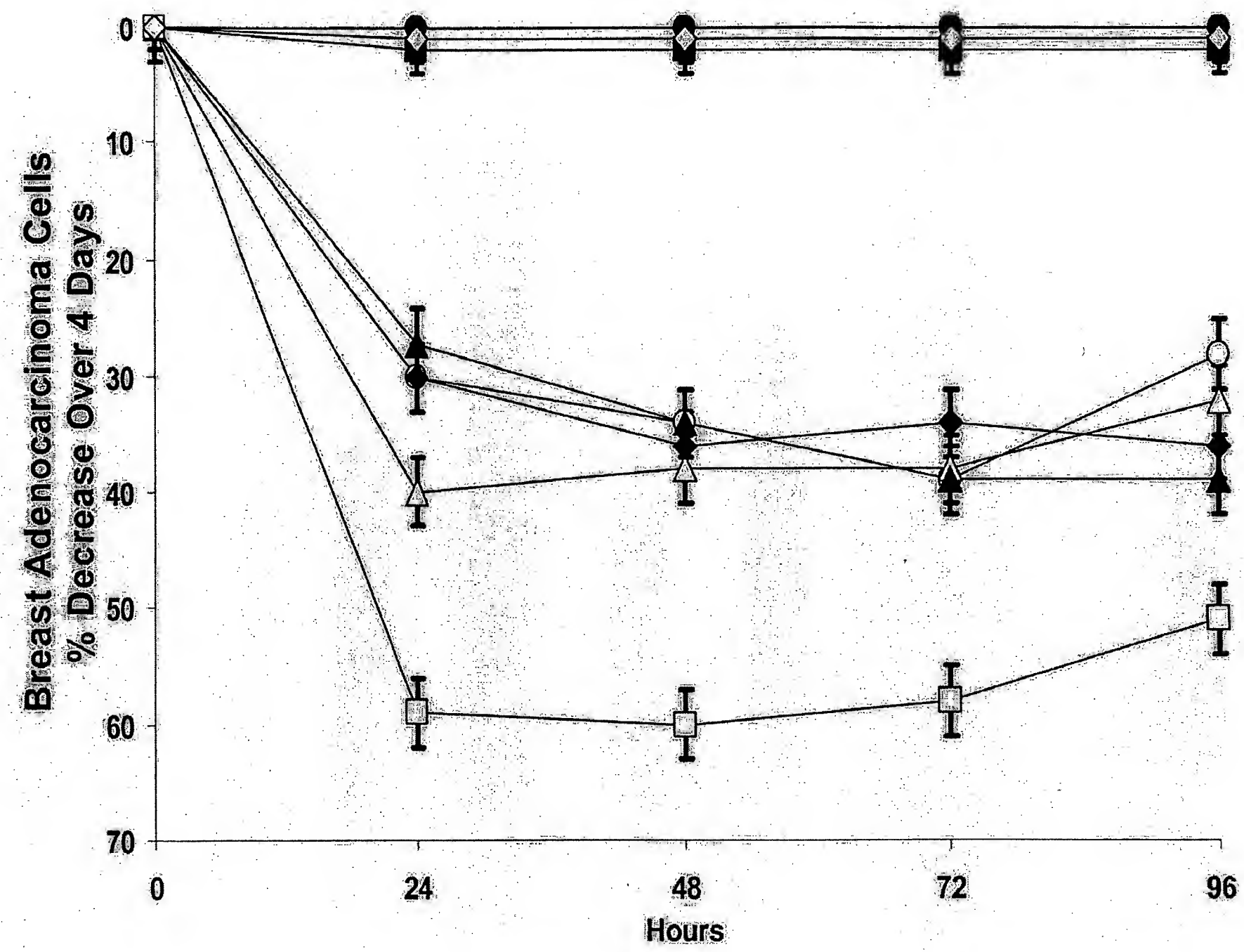
	Control	LANP	Vessel dilator	Kaliuretic peptide	ANP	BNP	CNP	cGMP
G_0-G_1 %	79	84	88	85	85	78	81	82
G_2-M %	2.5	4.1	4.8	5.5	1.5	4.7	6.3	6.0
S %	18	12	7	9	14	18	13	11
G_2-G_1 %	1.9	1.9	1.8	1.8	1.9	1.9	1.9	1.8
Debris %	7.9	13	11	14	13	8	11	12

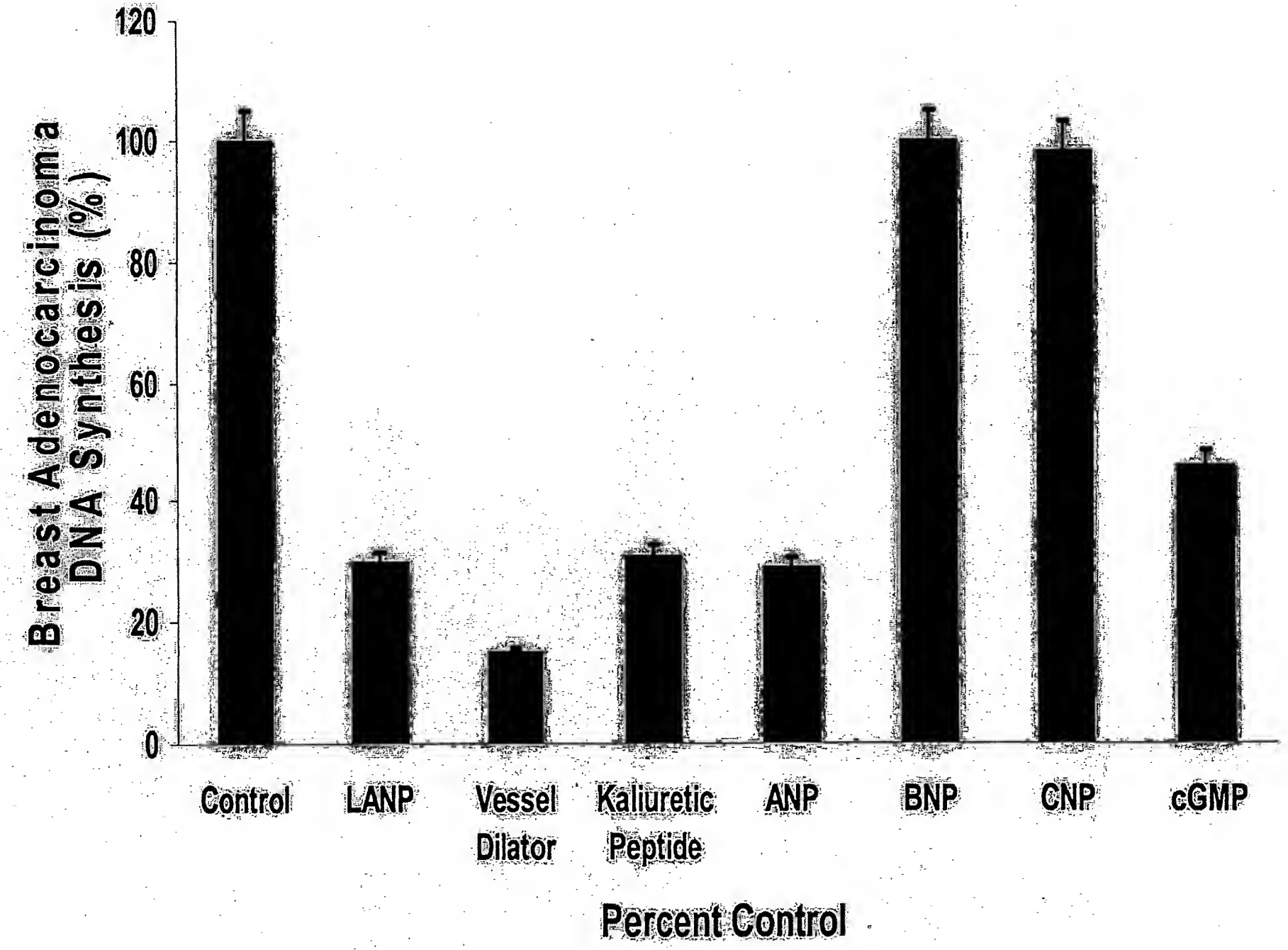
LANP = long acting natriuretic peptide, ANP = atrial natriuretic peptide, BNP = brain natriuretic peptide, CNP = C-type natriuretic peptide, and cGMP = 8-bromo-cyclic guanosine 3'-5' monophosphate. The decrease in the percent of cells in the S phase of the cell cycle was significant at $P<0.01$ for vessel dilator and kaliuretic peptide and significant at $P<0.05$ for LANP and cyclic GMP compared to untreated breast adenocarcinoma cells when evaluated by repeated measures of ANOVA. ANP's induced decrease of cells in G_2-M phase of the cell cycle was significant at $P<0.05$ when evaluated by repeated measures of ANOVA.

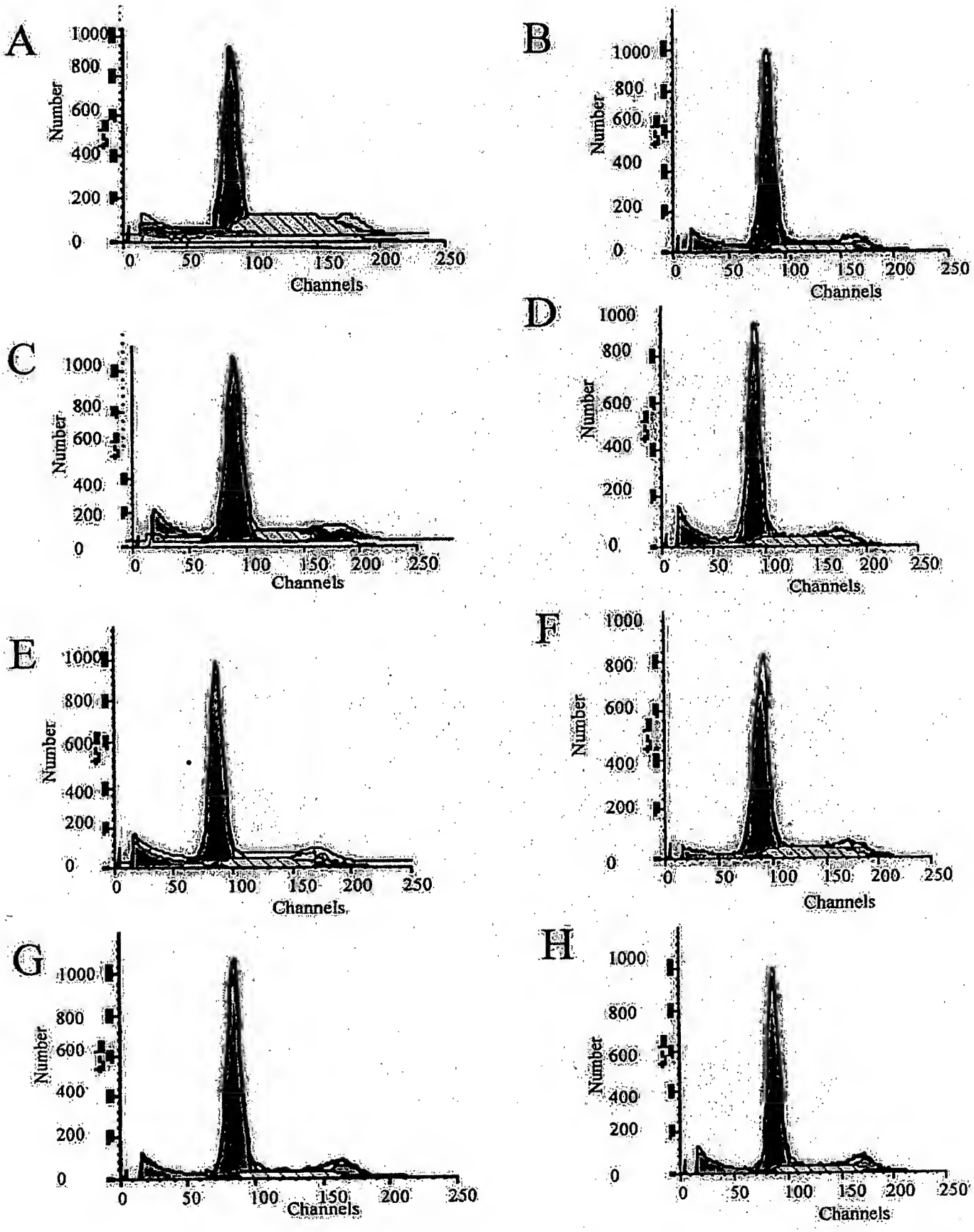
Abbreviations

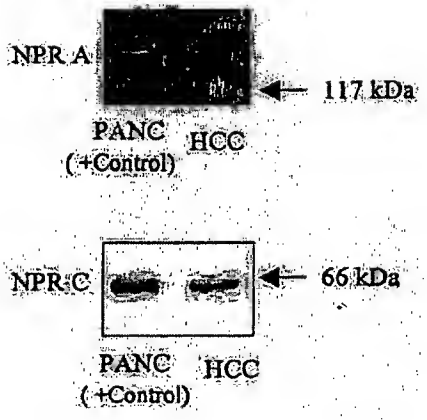
a.a.	amino acids
ANP	atrial natriuretic peptide
ATCC	American Type Culture Association
BNP	brain natriuretic peptide
BrdU	bromodeoxyuridine
CNP	C-type natriuretic peptide
C^0	centigrade
Cyclic GMP	cyclic 3',5' guanosine monophosphate
DNA	deoxyribose nucleic acid
EGF	epidermal growth factor
Fig	figure
G/L	gram per liter
G_2-M	G_2-mitotic (proliferative) phase of cell cycle
G_0-G_1	G_0, resting phase of cell cycle
mM/L	milli moles per liter
NPR-A	natriuretic peptide receptor-A (active)
NPR-C	natriuretic peptide receptor-C (clearance)
%	percent
RPMI	Rosewell Park Memorial Institute
rpm	revolutions per minute
S	synthetic phase of cell cycle
SHP	Shadyside Hospital, Pittsburg, PA


Control
LANP
Vessel Dilator
Kaliuretic Peptide
ANP
BNP
CNP
cGMP
0
10
20
30
40
50
60
70
Breast Adenocarcinoma
% Decrease in Cancer Cells


Breast Adenocarcinoma Cells
% Decrease Over 4 Days
0
10
20
30
40
50
60
70
0
24
48
72
96
Hours


Breast Adenocarcinoma
DNA Synthesis (%)
120
100
80
60
40
20
0
Control
LANP
Vessel Dilator
Kaliuretic Peptide
ANP
BNP
CNP
cGMP
Percent Control


A
B
C
D
E
F
G
H
Number
Channels
0
200
400
600
800
1000
0
50
100
150
200
250


NPR A
117 kDa
PANC
(+Control)
HCC
NPR C
66 kDa
PANC
(+Control)
HCC

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Four Peptide Hormones Decrease the Number of Human Small-Cell Lung Cancer Cells

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Running title: Four peptides decrease cancer cell number

Key words: Atrial natriuretic peptides; cancer cell proliferation; DNA synthesis; cyclic GMP

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ABSTRACT

Purpose: A family of six hormones, i.e., atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-natriuretic peptide (CNP), long acting natriuretic peptide (LANP), vessel dilator, and kaliuretic peptide were investigated for their ability to decrease the number of human small-cell lung cancer cells.

Experimental Design: These six hormones, each at their 1 μM concentrations, were evaluated for their ability to decrease the number and/or proliferation of small-cell lung cancer cells in culture for 24, 48, 72, and 96 hours.

Results: Within 24 hours, vessel dilator, LANP, kaliuretic peptide, ANP and their intracellular mediator cyclic GMP decreased the number of small-cell lung cancer cells 63%, 21%, 30%, 39%, and 31%, respectively. There was not any proliferation in the three days following this decrease in cell number. These same hormones decreased DNA synthesis 68% to 82%. BNP and CNP did not decrease the number of small-cell lung cancer cells or inhibit their DNA synthesis. Western blots revealed that the natriuretic peptide receptor-C was present in these cells.

Conclusions: Four peptide hormones significantly decrease the number of human small-cell lung cancer cells within 24 hours and inhibit their proliferation for at least 96 hours. Their mechanism of doing so involves inhibition of DNA synthesis mediated in part by cyclic GMP.

INTRODUCTION

Atrial natriuretic peptides consist of a family of peptides that are synthesized by three different genes then stored as three different prohormones [i.e., 126 amino acid (a.a.)[1] atrial natriuretic peptide (ANP), 108 a.a. brain natriuretic peptide (BNP), and 126 a.a. C-type natriuretic peptide (CNP) prohormones] (1-3). Within the 126 amino acid ANP prohormone are four peptide hormones whose main known biologic properties are blood pressure regulation and maintenance of plasma volume in animals (4-10) and humans (11-13). These peptide hormones, numbered by their amino acid sequences beginning at the N-terminal end of the ANP prohormone, consist of the first 30 a.a. of the prohormone (i.e., long acting natriuretic peptide, LANP), a.a. 31-67 (i.e., vessel dilator), a.a. 79-98 (kaliuretic peptide) and a.a. 99-126 (ANP) (3,14). The BNP and CNP genes, on the other hand, appear to each synthesize only one peptide hormone within their respective prohormones, i.e., BNP and CNP (2,15,16). Each of these peptide hormones circulates in healthy humans with vessel dilator and LANP concentrations in plasma being 15- to 20-fold higher than ANP, BNP and CNP (17-23).

We have recently found that vessel dilator, long acting natriuretic peptide, kaliuretic peptide and ANP decrease the number of human pancreatic adenocarcinoma cells in culture by 65%, 47%, 37% and 34%, respectively, within 24 hours (24). This decrease was sustained without any proliferation of the adenocarcinoma cells occurring in the three days following this decrease in number (24). ANP has also been reported to decrease the number of hepatoblastoma cells in culture (25). The mechanism of these peptide hormones' decrease in pancreatic adenocarcinoma cell number and antiproliferative effects included a 83% or greater inhibition of DNA synthesis but not

owing to enhanced apoptosis, i.e., programmed cell death (24). One of the known mediators of these peptide hormones' mechanism(s) of action, i.e., cyclic GMP inhibited DNA synthesis in these adenocarcinoma cells by 51% (24). When the four peptide hormones synthesized by ANP gene are given subcutaneously via osmotic pumps for one week in athymic mice with human pancreatic adenocarcinomas, these peptide hormones completely stop the growth of these adenocarcinomas (26). Three of the four peptides decreased the volume of the human pancreatic adenocarcinomas by 49%, 28%, and 11% while at the same time the volume of the adenocarcinomas in the placebo-treated animals increased 20-fold (26).

The present investigation was designed to determine if these four peptide hormones' effects are specific to adenocarcinomas or whether they might decrease the number of cancer cells of a different type of cancer i.e., small-cell lung cancer. Small-cell lung cancer was chosen for this investigation as it rarely is surgically curable (27) and, thus, if one or more of the peptide hormones do decrease the number of small-cell carcinoma cells, they may be a beneficial treatment for small-cell lung cancer as they do not have the side effects of the current cancer therapeutic agents (11-14). This investigation incorporates the atrial peptides made by all three genes within the heart, i.e., the above four peptide hormones plus BNP and CNP. When the four peptide hormones from the ANP gene were found to cause a significant decrease in the number of small-cell lung carcinoma cells, it was investigated whether the mechanism(s) of this decrease in the number of small-cell lung carcinoma cells and the ability of these peptides to inhibit further proliferation of these cancer cells after their decreased number was owing to inhibition of DNA synthesis. It was then determined whether their

intracellular mediator cyclic GMP could reproduce their effects on small-cell lung cancer cells and DNA synthesis.

MATERIALS AND METHODS

Small-Cell Lung Cancer Cells. A cell line (ATCC number CRL-2195, SHP-77; Shadyside Hospital, Pittsburg, PA) of human small-cell lung carcinoma cells was purchased from the American Type Culture Association (ATCC), Manassas, VA. This small-cell lung cancer cell line was derived in 1977 by E. R. Fisher, A. Palekar and J. D. Paulson from a non-encapsulated primary lung tumor from apical portion of the upper lobe of the left lung of a 54 year old Caucasian man (28). These cells, when injected into athymic mice, form tumors with a doubling time of 96 hours (28).

Culture of the Small-Cell Lung Carcinoma Cells. Propagation of these cells was in Roswell Park Memorial Institute (RPMI) 1640 medium with 2 mM/L-glutamine adjusted with addition of 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM of 90% sodium pyruvate and heat-inactivated 10% fetal bovine serum (Sigma Chemical Company, St. Louis, MO) at a temperature of 37°C as recommended by the ATCC. Cells were dispensed into new flasks with subculturing every 6-8 days. The growth medium was changed every three days.

Research Protocol

After the small-cell lung carcinoma cells were subcultured for 24 hours they were then seeded to coverslips in 24 well plates (Nunclon™, Denmark) with 1mL of the above RPMI media. After 24 hours, wells were washed twice with phosphate buffered saline to remove the fetal bovine serum. Removal of serum was done to completely remove all variables (EGF, etc.) present in serum in order that interpretation of any data obtained

would be straightforward. After 24 hours of serum deprivation, media volume was reduced to 250 µL per well with or without the respective peptide hormones at their 1µM concentrations (1% of this volume). Small-cell lung carcinoma cells were then incubated for various periods of time (24,48,72, and 96 hours). The number of small-cell lung carcinoma cells were then counted with a cell counter (Thomas Scientific®, Swedesboro, NJ) evaluating ten fields of the microscope slide at 40 x along the X-axis with an Olympus BH-2 microscope (Atlanta, Georgia). This evaluation was repeated on six separate occasions with the number of small-cell lung carcinoma cells reflecting 60 observations for each group, i.e., 60 observations for controls and 60 observations for each of the six groups with respective peptide hormones. The peptide hormones used in this investigation were from Phoenix Pharmaceuticals, Inc., Belmont CA.

Determination of DNA Synthesis. To investigate whether these peptide hormones were inhibiting DNA synthesis, bromodeoxyuridine (BrdU) incorporation into the small-cell lung carcinoma cells was utilized. BrdU was from BD Bioscience, San Jose, California. DNA synthesis and doubling of the genome take place during the synthetic or S phase (29,30). Bromodeoxyuridine is a thymidine analog incorporated into nuclear DNA during the S phase of the cell cycle (29-32). After 24 hours in culture with 1 µM of LANP, vessel dilator, kaliuretic peptide, ANP, BNP, or CNP, respectively, or with no peptide hormone (i.e., control), BrdU in a final concentration of 10 µM in the cell culture medium was added for 45 minutes - which is the time in which the cells are in the logarithmic phase of cell proliferation. For immunohistochemistry, a BrdU *in situ* detection kit from Becton Dickinson Immunocytochemistry Systems, San Jose, California, was utilized.

The incorporation of the BrdU stain into the nucleus was counted using a Nikon Inverted Diaphot-TMD Microscope (Tokyo, Japan). The number of stained nuclei were compared in the six peptide hormone groups to the positive control group. The negative control for these studies was provided by Becton Dickinson Immunocytochemistry Systems. To investigate DNA synthesis, BrdU incorporation by immunochemistry has been demonstrated to be equally good as ^{3}H-thymidine incorporation (31,32) and has the advantage that it provides high resolution (29,30).

Cyclic GMP Effects on DNA Synthesis. Cyclic GMP is one of the known mediators of the previously described biologic effects of these peptide hormones (4,33,34). All four of these peptides synthesized by the ANP gene-induced vasodilations of vasculature are mediated by increased cyclic GMP concentrations via enhancing guanylate cyclase activity (4,33,34). We have previously shown that each of these peptide hormones increase cyclic GMP while simultaneously dilating vasculature (4). 8 bromo-cyclic GMP reproduces these vasodilatory effects (33). For the present investigation of part of the mechanism of these peptide hormones' ability to inhibit DNA synthesis in small-cell lung carcinoma cells, 8-bromoguanosine 3',5'-cyclic monophosphate (i.e., 8-bromo cyclic GMP, Sigma) was utilized. 8-bromo cyclic GMP is a cell-permeable analog of cyclic GMP.

ANP Receptors in Small-Cell Lung Carcinoma Cells. When it was found that these ANPs decreased the number of human small-cell carcinoma cells, it was then evaluated whether small-cell lung carcinoma have ANP receptors to mediate these effects. Western blots of the natriuretic peptide receptors (NPR) A- and C- were performed as follows:

Western Blotting. Fifty micrograms of protein extract from small-cell lung carcinoma cells, measured by using the bicinchonic acid (BCA) protein assay kit (Pierce; Rockford, IL), was loaded onto each lane of a discontinuous 3.5/7.5% SDS-PAGE gel (Bio-Rad; Hercules, CA), separated by electrophoresis, and then transblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) for 85 min at 0.5 A in Towbin buffer. Blots were blocked for 1 hour at room temperature in a 5% solution of dry milk and then incubated for 1 hour in a 5% solution of bovine serum albumin (Fraction V; Fischer Scientific, Fair Lawn, NJ) in Tris-buffered saline that contained a 1:2,500 dilution of A035 polyclonal antibody directed against the COOH terminus of the NPR-A receptor protein (generously provided by Dr. David L. Garbers, University of Texas Southwestern, Dallas, TX) or containing Tris-buffered saline with a 1:400 dilution antibody to the NPR-C receptor (kindly provided by Dr. K. Omori, Osaka, Japan). After being washed with Tris-buffered saline, the membranes were incubated for 1 hour at room temperature in a solution of dry milk with a 1:15,000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG antibody (Amersham Life Sciences, Arlington Heights, IL). After a second washing with Tris-buffered saline, the bands were identified by enhanced chemiluminescence reagents (ECL Plus Kit; Amersham Pharmacia Biotech) and visualized in a luminescent image analyzer (model LAS-1000; Fujifilm, Tokyo, Japan). Specificity was revealed by the presence of a signal in rat lung (positive control) and absence of a signal with normal rabbit serum, rabbit IgG, and after preabsorption of the NPR-A antibody with NPR-A protein or preabsorption of the NPR-C antibody with NPR-C protein.

RESULTS

Decrease In Number of Small-Cell Carcinoma Cells by Four Peptide Hormones Synthesized by the ANP Gene. The number of small-cell lung carcinoma cells after 24 hours without the addition of any of the peptide hormones averaged 86 ± 9 cells per high-powered field when ten fields of the coverslip were evaluated at x 40 along the x-axis with an ©lympus BH-2 microscope (Atlanta, GA). This evaluation was repeated on six separate occasions with the above number reflecting sixty observations of the number of control small-cell lung carcinoma cells and sixty observations of each of the six groups with addition of one of the cardiac hormones (Fig. 1). The addition of 1 μM of long acting natriuretic peptide (LANP) for 24 hours decreased the number of small-cell lung carcinoma cells to 68 ± 6 i.e., a 21% decrease ($P<0.05$) in the number of small-cell carcinoma cells with the LANP (Fig. 1). Vessel dilator at 1 μM for 24 hours had an even more dramatic decrease (63%; $P<0.001$) in the number of these small-cell lung carcinoma cells (Figure 1). Vessel dilator decreased the number of cancer cells from 86 ± 9 cells to 32 ± 4. Kaliuretic peptide at 1 μM for 24 hours decreased the number of small-cell lung carcinoma cells 30% ($P<0.05$), i.e., to 60 ± 6 small-cell lung cancer cells (Fig. 1).

The number of small-cell lung cancer cells in culture decreased 39% ($P<0.01$) when exposed to atrial natriuretic peptide (1 μM) for 24 hours (Fig. 1). Brain natriuretic peptide and CNP, each at 1 μM, only decreased the number of small-cell lung carcinoma cells 0% and 4%, respectively, after 24 hours of incubation (not significant). Thus, with respect to their ability to inhibit the growth of small-cell carcinoma cells when these cells were exposed to identical concentrations of these six peptide hormones for

24 hours, vessel dilator > ANP > kaliuretic peptide> LANP > CNP > BNP. When the number of cells was examined immediately after the incubation of the respective peptide hormones within the cells, there was not any decrease in the number of cancer cells. In the wells with decreased number of cells secondary to the cardiac hormones, there was evidence of cellular debris.

Decreased Cellular Proliferation After Initial Decrease In Small-Cell Lung Carcinoma Cell Number. When small-cell lung cancer cells were exposed for longer periods of time e.g., 48, 72, and 96 hours to vessel dilator, LANP, kaliuretic peptide, ANP, BNP, and CNP each at 1 μM, there was a inhibition of proliferation of the small-cell lung carcinoma cells after the decrease in the number of these cancer cells at 24 hours by the peptide hormones from the ANP gene (Fig. 2). Thus, when exposed to vessel dilator, LANP, kaliuretic peptide and ANP for 48 hours the inhibition of the number of cancer cells compared to untreated small-cell carcinoma cancer cells was 54% ($P<0.001$), 20%, 21% and 24% ($P<0.05$ for these three peptides), respectively. At 72 hours and 96 hours, the decrease in number of small-cell carcinoma cells secondary to vessel dilator was 59% and 58% ($P<0.001$, Fig. 2). At both 72 and 96 hours, the number of small-cell carcinoma cells was reduced 21% ($P<0.05$ for both) compared to untreated small-cell lung cancer cells at these time periods. At 72 and 96 hours, the number of cancer cells with kaliuretic peptide present was decreased by 28% and 27%, respectively, compared to untreated small-cell lung carcinoma cells ($P<0.05$ for each) (Fig. 2). The number of small-cell lung cancer cells at 72 and 96 hours decreased secondary to ANP 30% and 32% ($P<0.05$ for both) compared to the number of small-cell lung cancer cells at these same time periods without the addition of any peptide

hormone (Fig. 2). Thus, proliferation was inhibited by these cardiac peptide hormones for three days after the initial decrease in cell number in the first 24 hours (Fig. 2). There was not any significant increase in proliferation of any of the cancer cells when exposed to these four peptide hormones for 1, 2, and 3 days after the initial decrease in number of the small-cell lung cancer cells within the first 24 hours. There was no significant decrease in small-cell lung cancer number secondary to BNP or CNP at 48, 72, or 96 hours (Fig. 2).

Cyclic GMP Decreases Small-Cell Lung Cancer Cell Number. Cyclic GMP at 1 μM decreased the number of human small-cell lung cancer cells 31% at 24 hours (Fig. 1). The decrease in number of human small-cell carcinoma cells at 48, 72, and 96 hours was 40%, 35%, and 32%, respectively ($P<0.05$ at all time points compared to control) (Fig. 2).

Inhibition of DNA Synthesis by Four Peptide Hormones. To help determine the mechanism of the small-cell lung carcinoma cells' decrease in number and decreased cellular proliferation by the above four peptide hormones, we next investigated whether their effects were due to an inhibition of DNA synthesis as they have been demonstrated to decrease DNA synthesis in pancreatic adenocarcinoma cells (24). Vessel dilator, LANP, kaliuretic peptide and ANP each at their 1 μM concentrations inhibited DNA synthesis when incubated with small-cell lung carcinoma cells for 24 hours by 82%, 75%, 68% and 78%, respectively ($P<0.001$ for each) (Fig. 3). There was not any significant decrease in DNA synthesis in the small-cell lung cancer cells secondary to BNP or CNP (Fig. 3).

Cyclic GMP Inhibits DNA Synthesis in Small-Cell Lung Cancer Cells. To help define the mechanism(s) for these peptide hormone's ability to decrease DNA synthesis, one of the known mediators of these peptides biologic effects, i.e., cyclic GMP (4,33,34) was investigated to determine if it could inhibit DNA synthesis in these same small-cell lung cancer cells. 8-bromo cyclic GMP decreased DNA synthesis in small-cell lung carcinoma cells by 50% ($P<0.01$) at its 1 μM concentration (Fig. 3).

NPR-C Receptor Is Present in Small-Cell Lung Cancer Cells. Small-cell lung cancer cell lines have been reported to have NPR-A receptors (35) but natriuretic receptors have never been evaluated in the small-cell lung cancer line utilized in the present investigation. It has never previously been investigated whether small-cell lung carcinomas have NPR-C receptors. When the small-cell lung carcinoma cells were evaluated by Western blots, the NPR-C receptor was demonstrated to be present (Fig. 4).

DISCUSSION

This investigation is the first evidence that vessel dilator, ANP, LANP and kaliuretic peptide can decrease the number of small-cell lung carcinoma cells. All four of these peptide hormones have been investigated for their effects on only one cancer previously, i.e., human pancreatic adenocarcinomas. Similar results were found with human pancreatic adenocarcinomas with each of the four peptide hormones synthesized by the ANP gene decreasing the number of human pancreatic adenocarcinoma cell during the first 24 hours 34% to 65% (24). Thus, these peptide hormones significantly decrease the number of cancer cells in both adenocarcinomas of the pancreas and small-cell lung carcinomas. The present investigation indicates,

therefore, that these peptide hormones significantly decrease the number of cancer cells of at least two different types of cancer within 24 hours. This information, plus the knowledge that one of these peptide hormones, i.e., ANP, decreases the number of hepatoblastoma cells in culture (25) suggests that these peptide hormones may have generalized anticancer effects, i.e., have the ability to decrease the number of cancer cells from a variety of different cancers. The ability of these four peptide hormones to completely stop the growth of human pancreatic adenocarcinomas *in vivo* with three of the four peptide hormones decreasing the volume of this human cancer in one week (26), highlights the potential clinical cancer treatment relevance of these peptide hormones.

Brain natriuretic peptide (BNP) and C-natriuretic peptide (CNP) did not have any significant effect on the number of the small-cell lung cancer cells. Thus, the effects on small-cell lung carcinoma cells with respect to atrial natriuretic peptides appear specific for the peptide hormones synthesized by the ANP gene as the peptides synthesized by the BNP and CNP genes had no significant effect on these cancer cells.

Vessel dilator was the most potent of these peptide hormones in decreasing the number of the small-cell lung cancer cells. Vessel dilator had significant ($P<0.001$) effects within 24 hours (63% decrease in number of small-cell lung cancer cells) and inhibited any further proliferation of the small-cell lung cancer cells from 24 to 96 hours ($P<0.001$; Fig. 2). Vessel dilator also decreased human pancreatic adenocarcinomas *in vitro* the most (24) and decreased human pancreatic adenocarcinoma tumor volume the most *in vivo* (26) suggesting that it has the most significant anticancer properties of these peptide hormones.

The other three peptide hormones synthesized by the ANP gene effects on decreasing the number of small-cell lung carcinoma cells was significant, however. Atrial natriuretic peptide decreased the number of small-cell carcinoma cells at 24 hours (39% decrease), which is more than its ability to decrease the number of pancreatic adenocarcinoma cells at 24 hours, i.e., 34% (24). Kaliuretic peptide, on the other hand, ability to decrease the number of small-cell lung carcinoma cells at 24 hours was not as good with small-cell lung carcinoma cells (30%) as with human pancreatic adenocarcinoma cells (37%). Thus, there appears to be a difference in these peptide hormones' ability to decrease cancer cell number depending upon the type of cancer. It should be noted that there was no decrease in the number of cells when examined immediately after addition of the respective peptide hormones indicating that the data obtained was not due to artifact. It is also important to note that cellular debris was present at 24 hours in the peptide hormone-treated small-cell lung carcinoma cell cultures suggesting that cellular necrosis was occurring.

Each of these peptide hormones inhibited 68% to 82% of the amount of DNA synthesis in these small-cell lung carcinoma cells. These findings suggest that one important mechanism of action of these peptide hormones to inhibit cancer cell number and their proliferation is via their ability to inhibit DNA synthesis. This finding is similar to the ability of these peptide hormones to inhibit DNA synthesis in human pancreatic adenocarcinoma cells (24), but considerably more significant than ANP's ability to inhibit DNA synthesis in hepatoblastoma cells (22%) (25).

With respect to the mechanism of how these peptide hormones inhibit DNA synthesis, one of the second messengers of their biologic effects, i.e., cyclic GMP

(4,32,33) was found using 8-bromo cyclic GMP to inhibit DNA synthesis up to 50% in the small-cell lung carcinoma cells. Cyclic GMP's mimicking the effects of these peptide hormones on DNA synthesis in the same cells suggests that cyclic GMP is one of the mediators of these peptide hormones' ability to inhibit DNA synthesis in small-cell lung cancer cells. The concentrations of cyclic GMP that inhibited DNA synthesis in the small-cell lung carcinoma cells are identical to the concentrations of cyclic GMP measured within tissues secondary to these peptide hormones (4). Further evidence that the cyclic GMP inhibition of DNA synthesis in small-cell lung cancer cells may be important for these peptide hormones anticancer growth effects is that when cyclic GMP was infused subcutaneously for a week in athymic mice with human pancreatic adenocarcinomas, it inhibited 95% of the growth of the human pancreatic adenocarcinoma compared to placebo-treated adenocarcinomas (26).

Brain natriuretic peptide (BNP) and C-natriuretic peptide (CNP)'s lack of biologic effects in these cancer cells with the knowledge of the present investigation that the NPR-C (i.e., clearance) receptor is present in these small-cell lung cancer cells suggests the possibility that the NPR-C receptor is clearing more BNP and CNP compared to ANP out of this cancer cell. Using 50μg of protein we were unable to detect the NPR-A receptor in the small-cell lung carcinoma cell line of the present investigation. The one previous investigation of small-cell lung cancer cell lines (but not the one of the present investigation) found that the NPR-A receptor was identified but was in such a low concentration that the number of binding sites could not be estimated (35). It is, thus, possible if one used a much larger amount of protein one might be able to detect an NPR-A receptor in the present small-cell lung cancer line, but the present

investigation and the previous investigation of the NPR-A receptor (35) indicate that this receptor is not abundant in small-cell lung cancers. The NPR-C receptor was demonstrated to be present for the first time in the present investigation in small-cell lung cancer cells. It is of interest that the small-cell lung carcinoma cells have developed an NPR-C receptor to clear ANP, BNP, and CNP out of the cell and, thus, decrease their effects.

Lung cancer is the leading cause of cancer death in both men and women in the United States, and this same trend is seen in many other countries (36). In the United States in 2004 it was estimated that lung cancer accounted for 31% of cancer deaths in men and 25% of cancer deaths in women (36). Small-cell lung cancers account for 20% to 25% of all lung cancers (27,37). Small-cell lung cancer differs from other lung cancers in that it metastasizes very early and rarely can be cured surgically (37). In the current management of small-cell lung cancers the majority of patients are treated first with chemotherapy plus radiotherapy but with this combination survival is only 20% at three years (37). There is an increased incidence of secondary cancers with the cisplatin- and cytoxan-based regimens for treating small-cell lung cancer and the chemotherapeutic regimens containing doxorubicin may induce cardiac myopathy (37). The data of the present investigation that these peptide hormones decrease the number of small-cell lung cancer cells suggests that these peptides may be a good addition to the therapeutic regimen for small-cell lung cancers since they do not cause secondary tumors or cardiac myopathy. Thus, their use would allow for the current anticancer chemotherapeutic agents to be used in a lower dose(s) to obtain the same effect. Utilizing one or more of these peptide hormones might also allow for a dose-escalation

of currently used chemotherapeutic agents as an important strategy for overcoming drug resistance (37). Thus, this dose-escalation would be less than the present escalation of current chemotherapeutic agents since the peptide hormones of the current investigation would decrease the number of cancer cells as demonstrated in the present investigation before this dose-escalation would begin. With less cells to kill, this dose escalation would not have to increase as much as in current clinical trials thereby achieving higher complete remission rates and increasing cures with less side effects as the total dose(s) of the chemotherapeutic agents to achieve cure this should be less if the above peptide hormones (without current anticancer agents' side effects, 4-16,24) are given first.

These peptide hormones which circulate normally in the human body (18-23) have no known cytoxic effects to normal cells (24) and only one known side effect (4-16). This side effect, i.e., hypotension, has only been observed with ANP and BNP and never with vessel dilator, LANP, or kaliuretic peptide (4-16). These last three peptide hormones with the best safety profile also decrease adenocarcinoma cells *in vitro* the most (24), small-cell lung cancer cells *in vitro* the most (present investigation), and decrease adenocarcinoma tumor volume *in vivo* the most of the atrial natriuretic peptides (26).

In addition to present adjunct chemotherapy for small-cell lung cancers causing a small incidence of secondary cancers and cardiac myopathy, presently utilized chemotherapy very commonly causes toxicity in the form of nausea, vomiting, alopecia, and myelosuppression (37). None of these toxicities occur with the atrial natriuretic peptide hormones (11-14). The data of the present investigation plus the knowledge

that these peptide hormones lack the above side effects (and have less side effects than all current utilized chemotherapeutic agents) suggests that they may be useful as adjunct chemotherapy for small-cell lung cancers even though they do not kill all of the small-cell lung cancer cells by themselves.

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FIGURE LEGENDS

Fig. 1. Decrease in human small-cell lung cancer cells after 24 hour exposure to 1 μM of long acting natriuretic peptide (LANP), vessel dilator, kaliuretic peptide, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). The decrease in small-cell lung cancer cells was significant at $P<0.001$ with vessel dilator, $P<0.01$ with ANP, and a $P<0.05$ with kaliuretic peptide and LANP when evaluated by repeated analysis of variance (ANOVA). There was no significant decrease in small-cell lung cancer cell number secondary to BNP or CNP when evaluated by ANOVA. The decrease in number of small-cell lung cancer cells secondary to vessel dilator was significantly greater ($P<0.05$) than the decrease secondary to any of the other natriuretic peptides when evaluated by ANOVA. Cyclic GMP (cGMP) decrease in small-cell lung cancer cells was significant at $P<0.05$ when evaluated by ANOVA.

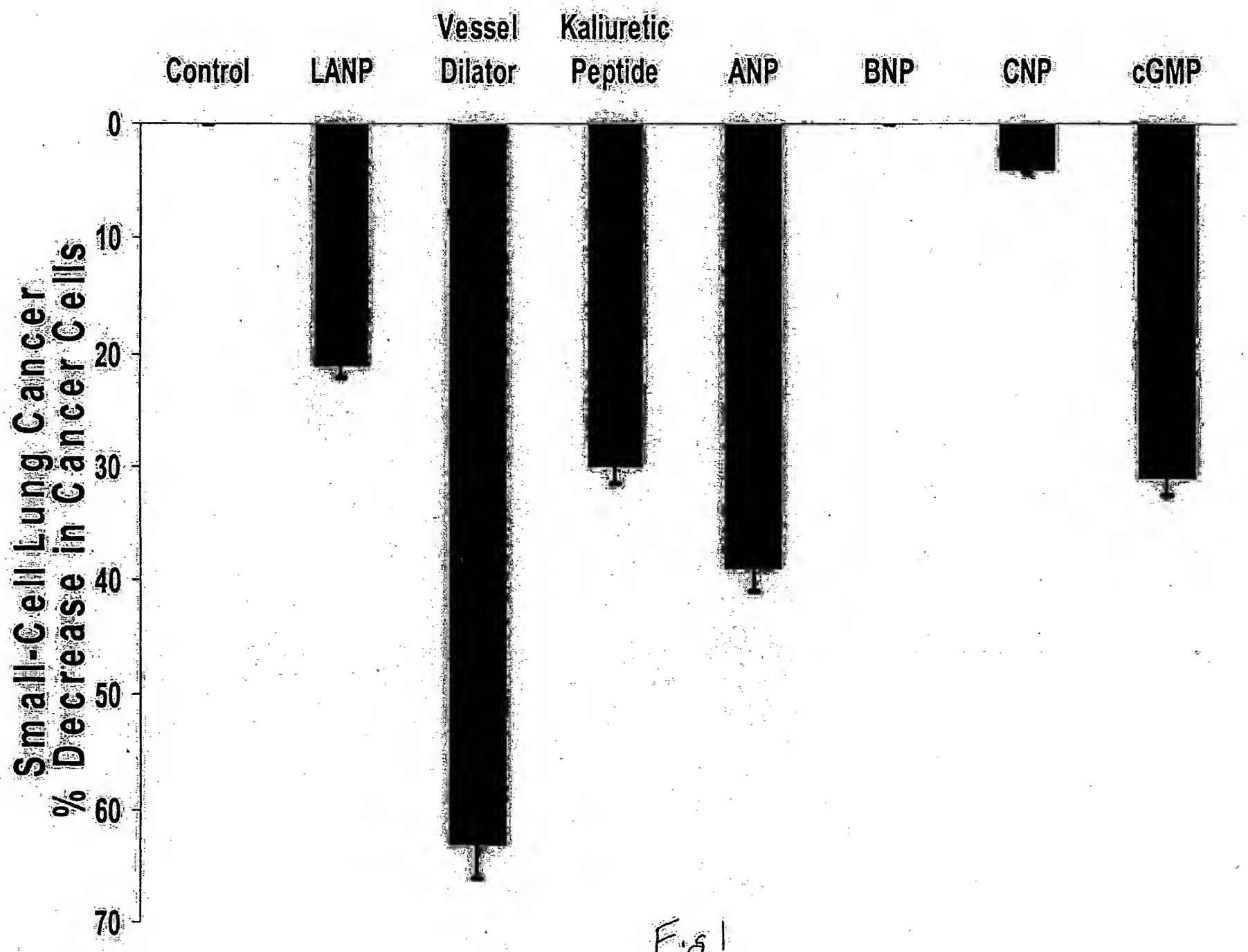
Fig. 2. Time course of decrease in human small-cell lung cancer cell number with 1 μM of vessel dilator (□), long acting natriuretic peptide (O, LANP), kaliuretic peptide (Δ), atrial natriuretic peptide (ANP, ▲), brain natriuretic peptide (BNP, ■), C-type natriuretic peptide (◇), respectively, and cyclic GMP (cGMP, 1 μM, ◆) compared to placebo-treated (●)small-cell lung cancer cells. The decrease in cancer cell number by ANP, LANP, kaliuretic peptide, and cGMP were significant at $P<0.05$ while the decrease secondary to vessel dilator was significant at $P<0.001$ at each time-point compared to placebo when evaluated by repeated analysis of variance (ANOVA). There was no significant decrease in small-cell lung cancer cell number with either BNP or CNP when evaluated by repeated ANOVA.

Fig. 3. Inhibition of DNA synthesis by vessel dilator, long acting natriuretic peptide (LANP), kaliuretic peptide, and atrial natriuretic peptide (ANP) in human small-cell lung cancer cells. This inhibition of DNA synthesis is illustrated as the percent of DNA synthesis occurring with the respective peptide hormones, each at 1 μM, versus the amount of DNA synthesis without the addition of any of these peptide hormones. The amount of inhibition of DNA synthesis by each of these peptide hormones was significant at $P<0.001$ when evaluated by repeated analysis of variance (ANOVA). 8-bromo cyclic GMP at its 1 μM inhibited DNA synthesis 54% in small-cell lung cancer cells ($P<0.01$). BNP and CNP, each at 1 μM, had no significant effect on DNA synthesis when evaluated by repeated ANOVA.

Fig. 4. NPR A- and C-receptors evaluation human small-cell lung cancer cells. Western blot analysis with 1:2500 dilution of A035 polyclonal antibody directed against the COOH terminus of the natriuretic peptide A-receptor (NPR-A) and 1:4000 dilution of antibody to the NPR-C receptor. The upper graph demonstrates the positive rat lung control (PANC) for the NPR-A receptor without evidence of the NPR-A receptor in the small-cell lung cancer (SHP-77). The lower graph demonstrates the NPR-C receptor at 66 kiloDaltons (kDa) in the human small-cell lung cancer cells (SHP-77) as well as in the postitve control (left panel).

Abbreviations

a.a.	amino acids
ANP	atrial natriuretic peptide
ATCC	American Type Culture Association
BNP	brain natriuretic peptide
BrdU	bromodeoxyuridine
CNP	C-type natriuretic peptide
C^0	centigrade
Cyclic GMP	cyclic 3',5' guanosine monophosphate
DNA	deoxyribose nucleic acid
EGF	epidermal growth factor
Fig	figure
G/L	gram per liter
G_2-M	G_2-mitotic (proliferative) phase of cell cycle
G_0-G_1	G_0, resting phase of cell cycle
mM/L	milli moles per liter
NPR-A	natriuretic peptide receptor-A (active)
NPR-C	natriuretic peptide receptor-C (clearance)
%	percent
RPMI	Rosewell Park Memorial Institute
rpm	revolutions per minute
S	synthetic phase of cell cycle
SHP	Shadyside Hospital, Pittsburg, PA


Control
LANP
Vessel Dilator
Kaliuretic Peptide
ANP
BNP
CNP
cGMP
0
10
20
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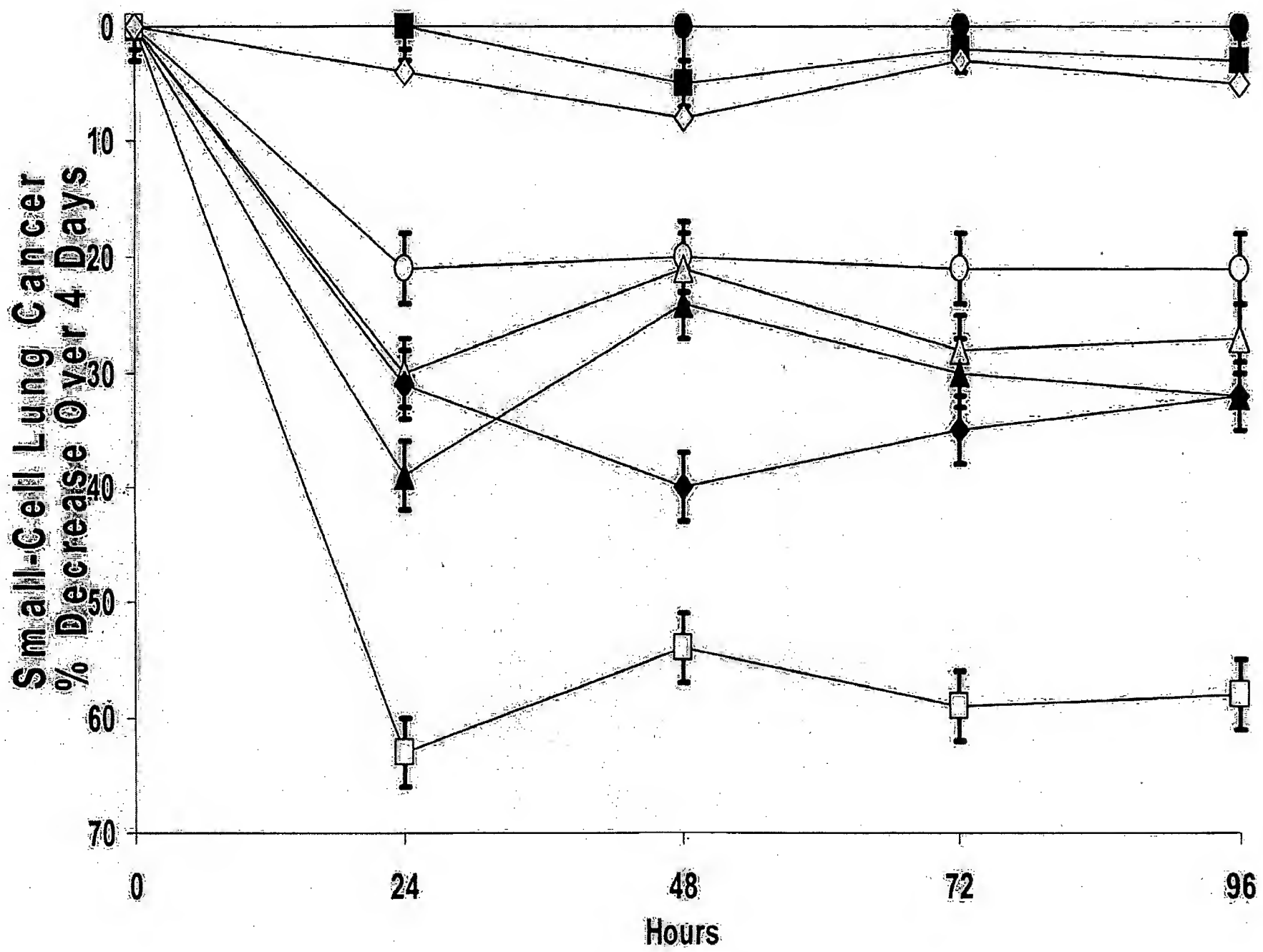
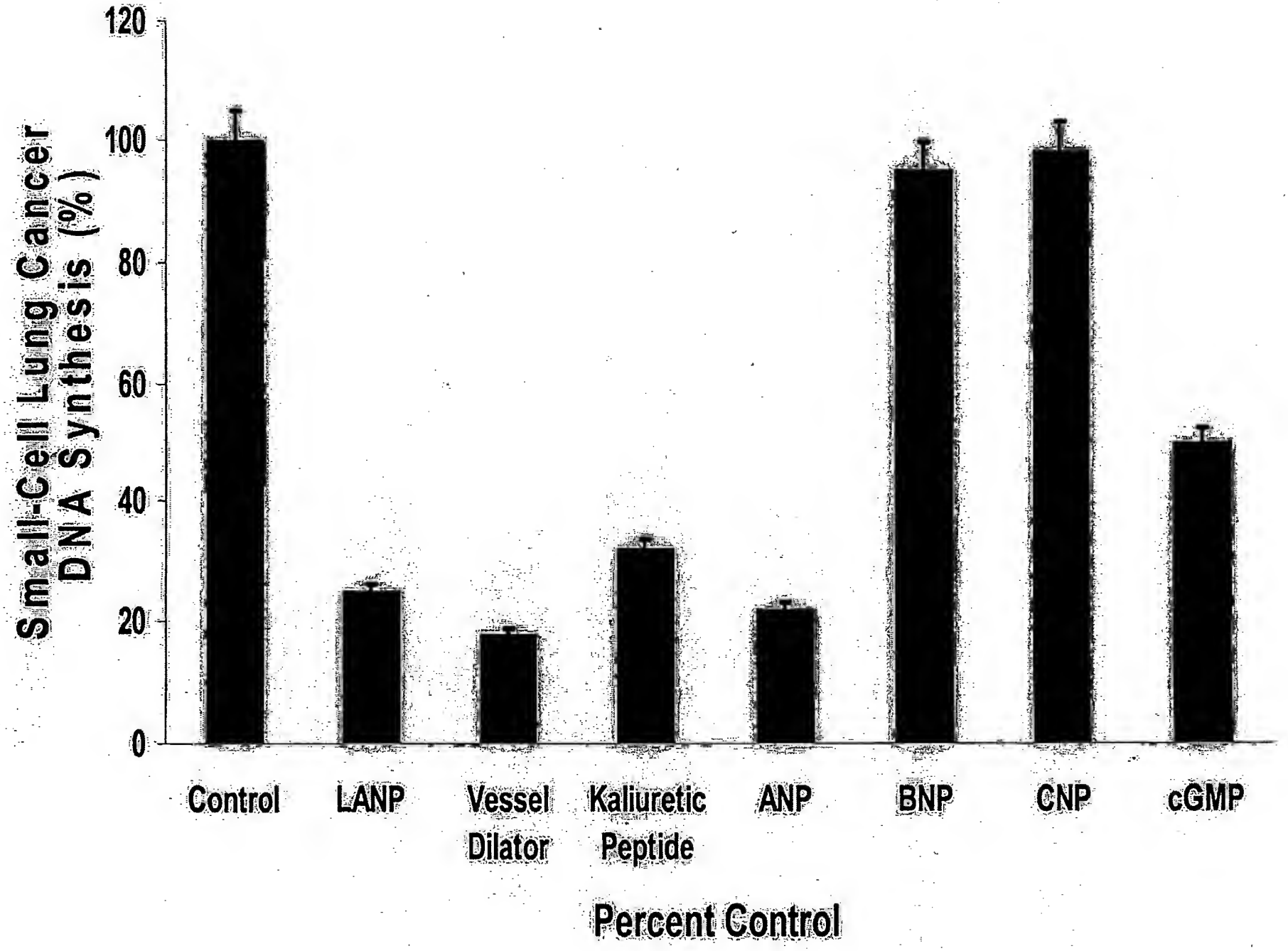
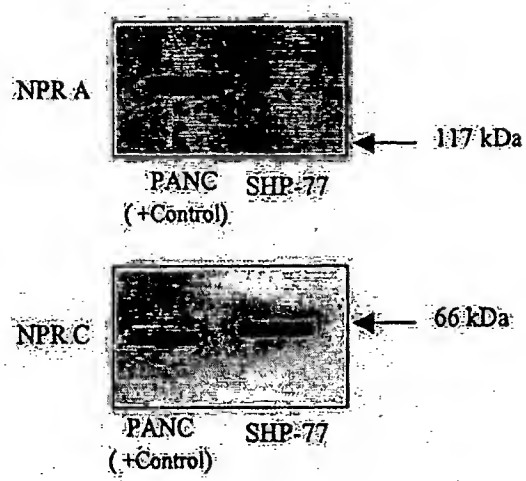
40
50
60
70
Small-Cell Lung Cancer
% Decrease in Cancer Cells

Fig 1


Small-Cell Lung Cancer
% Decrease Over 4 Days
0
10
20
30
40
50
60
70
0
24
48
72
96
Hours


Small-Cell Lung Cancer
DNA Synthesis (%)
120
100
80
60
40
20
0
Control
LANP
Vessel Dilator
Kaliuretic Peptide
ANP
BNP
CNP
cGMP
Percent Control


NPR A
117 kDa
PANC
(+Control)
SHP-77
NPR C
66 kDa
PANC
(+Control)
SHP-77

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LOCALIZATION OF FOUR PEPTIDES IN ADENOCARCINOMAS

Immunocytochemical Localization of Atrial Natriuretic Peptide, Vessel Dilator, Long Acting Natriuretic Peptide, and Kaliuretic Peptide in Human Pancreatic Adenocarcinomas

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Abstract

We recently found that four peptide hormones synthesized by the same gene completely inhibit the growth of human pancreatic adenocarcinomas in athymic mice. The present immunocytochemical investigation was designed to determine where in the adenocarcinomas these peptide hormones localize. Atrial natriuretic peptide, vessel dilator, long acting natriuretic peptide and kaliuretic peptide localized to the cytoplasm and nucleus of the human pancreatic adenocarcinomas which is consistent with their ability to decrease DNA synthesis in the nucleus of this cancer. In this first investigation of where these peptide hormones with anticancer effects localize in any cancer, these peptide hormones also localized to the endothelium of capillaries and fibroblasts within these cancers. Conclusion: This is the first demonstration of growth-inhibiting peptide hormones localizing to the nucleus where they inhibit DNA synthesis and may interact with growth-promoting hormones which localize there as the etiology of their ability to inhibit the growth of adenocarcinomas both in vitro and in vivo.

Key words: Atrial Natriuretic peptides; Immunocytochemistry; pancreatic adenocarcinomas

Introduction

A family of peptide hormones referred to as atrial natriuretic peptides (ANPs) are synthesized within the heart and stored in the atrial myocyte as prohormones for rapid release in response to stimuli (Brenner et al. 1990; Vesely 2003). The ANP gene synthesizes a 126 amino acid (a.a.) prohormone, which contains four peptide hormones consisting of a.a. 1-30 (i.e., long acting natriuretic peptide, LANP), a.a. 31-67 (vessel dilator), a.a. 79-98 (kaliuretic peptide) and atrial natriuretic peptide (ANP, a.a. 99-126 of this prohormone) (Brenner et al. 1990; Vesely 2002). Known biologic properties of these four peptide hormones include blood pressure lowering, diuresis, enhanced sodium and/or potassium excretion when infused into healthy animals (Vesely et al. 1987; Martin et al. 1990; Gunning et al. 1992; Benjamin and Peterson, 1995; Clark et al. 2000) and humans (Vesely et al. 1994a,b; 1998, 1999; Nasser et al. 2001).

We have found that vessel dilator, LANP, kaliuretic peptide and ANP decrease the number of human pancreatic adenocarcinoma cells in culture by 65%, 47%, 37% and 34%, respectively, within 24 hours (Vesely et al. 2003). This decrease was sustained without any proliferation of the adenocarcinoma cells occurring in the three days following this decrease in number (Ibid). The mechanism of these peptide hormones' decrease in cancer cell number and antiproliferative effects was a 83% or greater

inhibition of DNA synthesis but not owing to enhanced apoptosis, i.e., programmed cell death (ibid). One of the known mediators of these peptide hormones' mechanism(s) of action, i.e., cyclic GMP, inhibited DNA synthesis in these adenocarcinoma cells by 51% (ibid).

In vivo, vessel dilator (139 ng $min^{-1}kg^{-1}$ of body weight) infused for 14 days completely stopped the growth of the human pancreatic adenocarcinomas in athymic mice (n=14) with a decrease in their tumor volume, while the tumor volume increased 69-fold ($P<0.001$) in the placebo (n=30)-treated mice (Vesely et al. 2004). When the four peptide hormones synthesized the ANP gene (each at 1.4 μg $min^{-1}kg^{-1}$ body weight) were infused for four weeks, vessel dilator, long acting natriuretic peptide and kaliuretic peptide in addition to completely stopping the growth of this human pancreatic cancer also decreased tumor volume after one week by 49%, 28%, and 11% with a one- and 20-fold increase in the tumor volume in ANP- and placebo-treated mice (ibid).

The present investigation was designed to determine where these peptide hormones localize within human pancreatic cancers. Where these peptide hormones localize within any cancer has never previously been investigated.

Materials and Methods

Tissue Samples:

The human pancreatic adenocarcinomas growing in athymic mice were removed after separate four-week infusions via Alzet Model 2004 osmotic pumps (Duret Corporation, Cupertino, CA) of vessel dilator, long acting natriuretic peptide (LANP), kaliuretic peptide, and ANP with peptide each at a concentration of 1.4 $\mu g\ min^{-1} kg^{-1}$ body weight) (Vesely et al. 2004). The Alzet Model 2004 osmotic pump for mice delivers all of its contents (200 μL) over 28 days at a rate of 0.25 $\mu L\ h^{-1}$ and then stops pumping. Untreated human pancreatic adenocarcinomas were also examined four weeks after they had became palpable, at which time their volume had increased 299-fold from when they were first palpated. These treated and untreated pancreatic adenocarcinomas were fixed in zinc-formalin for 24 hours, paraffin-embedded and subsequently processed with routine techniques and IHC analysis described previously from our laboratory (Ramirez et al. 1992; Saba et al. 1993). Several tumors of animals treated with vessel dilator, LANP, kaliuretic peptide, and ANP, respectively, as well as several untreated human pancreatic adenocarcinomas were evaluated.

Immunohistochemistry:

After deparaffinization and rehydration with phosphate-buffered saline (PBS), pH 7.2, 3 μm sections were stained for vessel dilator, long acting natriuretic peptide (LANP), kaliuretic peptide, and atrial natriuretic peptide (ANP) by the avidin-biotin peroxidase complex (ABC) technique (Hsu et al. 1981) using a Vectastain kit® (Vector Labs, Burlingame, CA, USA). Sections were incubated for 1 hour at 24°C in a humidity chamber with the respective primary antibody diluted (1:200-1:1000) with PBS, and then after rinsing, incubated again in the humidity chamber for 30 min with biotinylated antibody. The illustrations in this manuscript are all at the 1:800 dilution of the respective primary antibodies. The slides were then rinsed followed by a final incubation for 30 min in ABC in a humidity chamber. Controls for the immunoperoxidase staining included: (a) substitution of the primary antibody with normal rabbit serum and (b) preincubation of the primary antibody with excess vessel dilator, LANP, kaliuretic peptide and/or ANP in their respective immunoperoxidase assays for 24 hours at 37°C. These respective human peptide hormone antisera for immunohistochemistry were from Peninsula Laboratories (Belmont, CA, USA).

Cross reactivity of vessel dilator, LANP, kaliuretic peptide, and ANP:

Cross reactivities of the various human antisera raised in rabbits to determine their specificity were determined by comparative radioimmunoassays utilizing the human form of pure synthetic peptides vessel dilator, LANP, kaliuretic peptide, and ANP synthesized and assayed by Peninsula Laboratories, Inc (Belmont, CA, USA). LANP antisera cross reactivity with pure synthetic LANP was 100% while its cross reactivity with ANP and vessel dilator was 0%. Vessel dilator antisera had 100% cross reactivity with pure synthetic vessel dilator but 0% cross reactivity with LANP and/or ANP. The antisera to ANP had 100% cross reactivity with ANP, and 0% cross reactivity with either LANP or vessel dilator. Kalluretic peptide antisera had 100% cross reactivity with human kaliuretic peptide but 0% cross-reactivity with LANP, vessel dilator or ANP.

Results

Immunoperoxidase staining of vessel dilator was very strong (+++) in the cytoplasm of the human adenocarcinoma cells (Fig. 1A) compared to control adenocarcinoma cells (Insert Fig. 1D) which did not receive a vessel dilator infusion in vivo. Essentially all of the cytoplasm of the pancreatic adenocarcinoma cells had strong staining with vessel dilator (Fig. 1A). The nucleus of the adenocarcinoma cells had weaker but still strong vessel dilator immunoperoxidase staining. Vessel dilator also localized (+++) to the endothelium of the small capillaries invading the human pancreatic adenocarcinoma. On histological (H&E) staining there were not any large blood vessels (arteries, etc) within these tumors but numerous small capillaries invading this tumor could be visualized (Fig. 2). In the H & E evaluation one can observe mitosis occurring within the cancer cells with their nuclei clearly discernable (Fig. 2). Each of the tumors had necrotic centers in both the treated and untreated adenocarcinomas. Vessel dilator also localized to the fibroblasts within the adenocarcinomas where the cytoplasm but not the nuclei of the fibroblasts stained positive for vessel dilator (Fig. 1A).

Long acting natriuretic peptide (LANP) had a slightly stronger (++++) immunoperoxidase staining of the cytoplasm but similar intensity (+++) of immunoperoxidase staining of the nucleus of the human

pancreatic adenocarcinomas compared to vessel dilator (Fig. 1B). Strong (++++) LANP immunoperoxidase staining was noted in the endothelium in the small capillaries in the adenocarcinomas (Fig. 1B). LANP staining of the fibroblasts within this tumor was more intense (++++) than with vessel dilator (Fig. 1B).

Kaliuretic peptide immunoperoxidase staining in the adenocarcinomas infused with kaliuretic peptide had a decreased intensity compared to the other peptide hormones (Table 1 and Fig. 1C). Kaliuretic peptide, however, localized to the same structures within the human pancreatic adenocarcinomas (Fig. 1C). Thus, kaliuretic peptide immunoperoxidase staining (++) localized to the nucleus of the cancer cells similar to that observed with vessel dilator and LANP (Fig. 1C). Staining of the cytoplasm of the human pancreatic adenocarcinoma cells with kaliuretic peptide was less than that observed with vessel dilator but there was definite localization of kaliuretic peptide to the cytoplasm. Kaliuretic peptide also localized to the endothelium of the small capillaries and to fibroblasts (Fig. 1C) with an intensity similar to that of its localization to cytoplasm.

ANP had very strong immunoperoxidase (++++) localization to the nucleus and cytoplasm of the human pancreatic cells (Fig. 1D). ANP had slightly stronger immunoperoxidase staining of the nucleus than each of

the other peptide hormones (Table 1). ANP also had a very strong (+++) localization to the endothelium of the capillaries invading this cancer. Strong (++++) ANP immunoperoxidase staining was also present in the fibroblasts in this tumor (Fig. 1D). The immunohistochemical data are summarized in Table 1.

There was not any immunoperoxidase staining with vessel dilator, LANP, kaliuretic peptide or ANP in the human adenocarcinomas when their respective primary antisera were either substituted with normal rabbit serum (Insert Fig. 1D) or when the primary antibody was preincubated with an excess of vessel dilator, LANP, kaliuretic peptide, and/or ANP in their respective immunoperoxidase assays for 24 hours at 37°C.

Discussion

This is the first demonstration of the localization of four peptide hormones synthesized by the atrial natriuretic peptide gene in any cancer. Human pancreatic adenocarcinomas were chosen as the first cancer to be investigated because persons with pancreatic adenocarcinoma have the lowest five-year survival rate (1%; with a median survival of only 4 months) of all common human malignancies (Ptichumoni 1998; Wolff et al. 2000). Surgery and chemotherapy extend survival by a few months but the above four-month median survival and five-year survival rate includes those persons treated with surgery and chemotherapy (Ptichumoni 1998; Wolff et al. 2000). The human pancreatic carcinomas of the present investigation had their growth completely stopped by each of the four peptide hormones of this investigation and vessel dilator decreased the volume of these adenocarcinomas 49% after each were infused for a week subcutaneously (Vesely et al. 2004). Each of the four peptide hormones in the present investigation had a strong localization in the nucleus of the cancer cells. This information correlates with the knowledge that each of these peptide hormones strongly inhibit DNA synthesis (83% to 91% decrease) within the human pancreatic adenocarcinomas of the present investigation (Vesely et al. 2003) as the nucleus is the site of DNA synthesis. Further correlation of the findings of the localization of these

peptide hormones in the nucleus of the human pancreatic adenocarcinomas in the present investigation are flow cytometry studies demonstrating that these peptide hormones decrease the number of adenocarcinoma cells in synthetic (S) phase of the cell cycle i.e., where DNA synthesis occurs (Vesely et al. unpublished observation).

Vessel dilator, LANP, kaliuretic peptide, and ANP had a very strong localization to the cytoplasm of these cancer cells. We had hypothesized that if these peptide hormones were reaching the nucleus to inhibit DNA synthesis, they should also be abundantly present in the cytoplasm after binding to the cell surface receptors on plasma membranes of the cancer cells. These peptides are known to bind to specific receptors in the plasma membranes of normal and cancer cells (Vesely et al. 1987, 1990, 1992). Once ANP binds to its receptor, the receptor internalizes and ANP was then thought to be degraded with the receptor(s) recycling to the plasma membrane (Hirata et al. 1985; Napier et al. 1986; Hughes et al. 1987; Morl et al. 1988). Part of the cytoplasmic demonstration of these peptide hormones within the cancer cells may be the atrial natriuretic peptides (ANPs) attached to their receptor(s) which is being internalized. However, the intense cytochemical localization throughout the cytoplasm and the new knowledge of the present investigation that these peptides also localize in the nucleus suggests that these peptides are not all being

degraded in the cytoplasm as previously thought but rather are traveling through the cytoplasm to reach the nucleus to directly inhibit DNA synthesis. These peptide hormones do have an intracellular mediator within the cytoplasm, i.e., cyclic GMP (Brenner et al. 1990; Vesely 1997). Cyclic GMP inhibits DNA synthesis in these cancer cells, but only to about half of the extent of vessel dilator (Vesely et al. 2003). The new knowledge of the present investigation that the peptide hormones are reaching the nucleus via the cytoplasm suggest that these peptide hormones may be directly inhibiting DNA synthesis and decreasing cell cycle progression (with 62% less cells in the S phase) as well as indirectly inhibiting DNA synthesis via the intracellular messenger cyclic GMP.

Cells that are targets for the action of peptide hormones and/or growth factors express specific receptors for these peptides on the surface of their plasma membranes (Hirata et al. 1985). Receptors provide the underlying specificity and sensitivity for hormone/growth factor activity. Upon binding to its receptor, respective peptides are internalized into the target cell by receptor-mediated endocytosis (Hirata et al. 1985). The newly formed endocytic vesicles provide the structural basis for the subsequent vesicular transport of the receptor-ligand complexes to the lysosomal compartment of the target cell. Lysosomal degradation of the receptor-ligand complexes is thought to account for the down-regulation of

receptors and the self-limiting response of the target cell to the peptide hormone-induced stimulation (Hirata et al. 1985). Because peptide hormones have been thought to be degraded, events initiated at the cell surface have been assumed to be responsible for the subsequent biological response of the target cell. However, a number of laboratories have shown an association of peptide hormones and/or growth factors with the nucleus of target cells (Burwen and Jones 1987). Included in this list are insulin, luteinizing hormone releasing hormone, human chorionic gonadotrophin, nerve growth factor, epidermal growth factor and platelet derived growth factor (Burwen and Jones 1987). This association has been demonstrated by localization of the peptide hormones in the nuclei and detection of specific binding sites for the peptide hormones on or in nuclei. The accumulated evidence has been largely ignored because it does not fit the current conceptual framework that mandates the lysosomal destruction of all endocytosed peptides (Burwen and Jones 1987). Further evidence of this point is that current textbooks of endocrinology do not mention peptide hormones localizing to nucleus, i.e., they only discuss thyroid and steroid hormones localizing to the nucleus (Lazar 2003).

That peptide hormones localize to the nucleus requires that an alternative transport pathway exists, resulting in delivery of endocytosed

peptide to the nucleus instead of lysosomes. In addition, a direct association between a peptide hormone and the nucleus suggests that these peptides may exert at least some of their biological effects directly at the nuclear level (Burwen and Jones 1987). It is important to note in this regard that all of the previously reported peptide hormones that localize to the nucleus have been growth-promoting hormones (Burwen and Jones 1987). The present investigation is the first demonstration that we are aware of that growth-inhibiting peptide hormones localize to the nucleus, where they can directly interact to inhibit the effects of the growth-stimulating hormones as well as act via their demonstrated direct decrease of DNA synthesis in the nucleus (Vesely et al. 2003).

In the present investigation these peptide hormones also localized to the endothelium of the blood vessels growing into this cancer. This finding is similar to previous finding that these peptide hormones localized to the endothelium of blood vessels of normal kidneys (Ramirez et al. 1992; Saba et al. 1993). In normal tissues these peptides localize to the endothelium of larger arteries and veins (Ramirez et al. 1992; Saba et al. 1993), but in the present investigation, when examined by H & E staining (Fig. 2), there was not any large blood vessels growing into these tumors but rather it was only capillaries that were invading this cancer. Within the capillaries, it was the endothelium that had immunoperoxidase staining of

these peptide hormones (Fig. 1). The fact that no large blood vessels had grown into these adenocarcinomas helps explain the observation on H & E examination that the centers of each of these tumors were necrotic. This finding is apparently due to these tumors outgrowing their blood supply (i.e., no large blood vessels were present) rather than due to an effect of these peptide hormones per se as the untreated adenocarcinomas had equally as much necrosis in the center of their tumors as the vessel dilator, LANP, kaliuretic peptide and ANP-treated tumors.

LANP, vessel dilator, ANP and kaliuretic peptide also localized to the fibroblasts within the adenocarcinomas in the present investigation. It could not be determined with certainty in the present investigation whether these peptide hormones were being synthesized by the fibroblasts or whether they localized to the fibroblasts after their infusion. ANP prohormone messenger RNA is present in fibroblasts, which indicates that vessel dilator, LANP, kaliuretic peptide, and ANP, which are derived from the ANP prohormone, are synthesized by fibroblasts (Kwano et al. 2000). The fact that there was no localization of these peptide hormones to fibroblasts in the untreated adenocarcinomas would suggest that their localization to fibroblasts was due the infusion of the respective peptide hormones.

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Figure Legends

Figure 1. Immunoperoxidase localization of vessel dilator, long acting natriuretic peptide (LANP), kaliuretic peptide and atrial natriuretic peptide (ANP) within human pancreatic adenocarcinomas with each of these peptide hormones strongly localizing to cytoplasm (Cyt), nucleus (N), endothelium (E) and fibroblasts (F). The light blue stain in the fibroblasts is the nuclei of the fibroblasts. A) Vessel dilator-treated, B) LANP-treated, C) Kaliuretic peptide-treated, D) ANP-treated. The negative control is as an insert in D. Primary antibody of each peptide was diluted 1:800. Original magnification x 60.

Figure 2. Histology (H&E) of human pancreatic adenocarcinoma illustrating capillaries (but not large blood vessels) growing into adenocarcinoma. CAP = capillaries, N = nucleus of human pancreatic adenocarcinoma cell, M = mitosis within adenocarcinoma cell, and F = fibroblasts with human pancreatic adenocarcinoma. Original magnification x 60.

Table 1. Immunohistochemical localization of vessel dilator, long acting natriuretic peptide (LANP), kaliuretic peptide, and atrial natriuretic peptide (ANP) in human pancreatic adenocarcinomas.

	Vessel dilator	LANP	Kaliuretic peptide	ANP
Cytoplasm	+++	++++	++	++++
Nucleus	+++	+++	++	++++
Endothelium	+++	++++	++	++++
Fibroblasts	+++	++++	++	++++

Immunoperoxidase staining graded 0 to +++, with ++++ being the strongest staining observed.

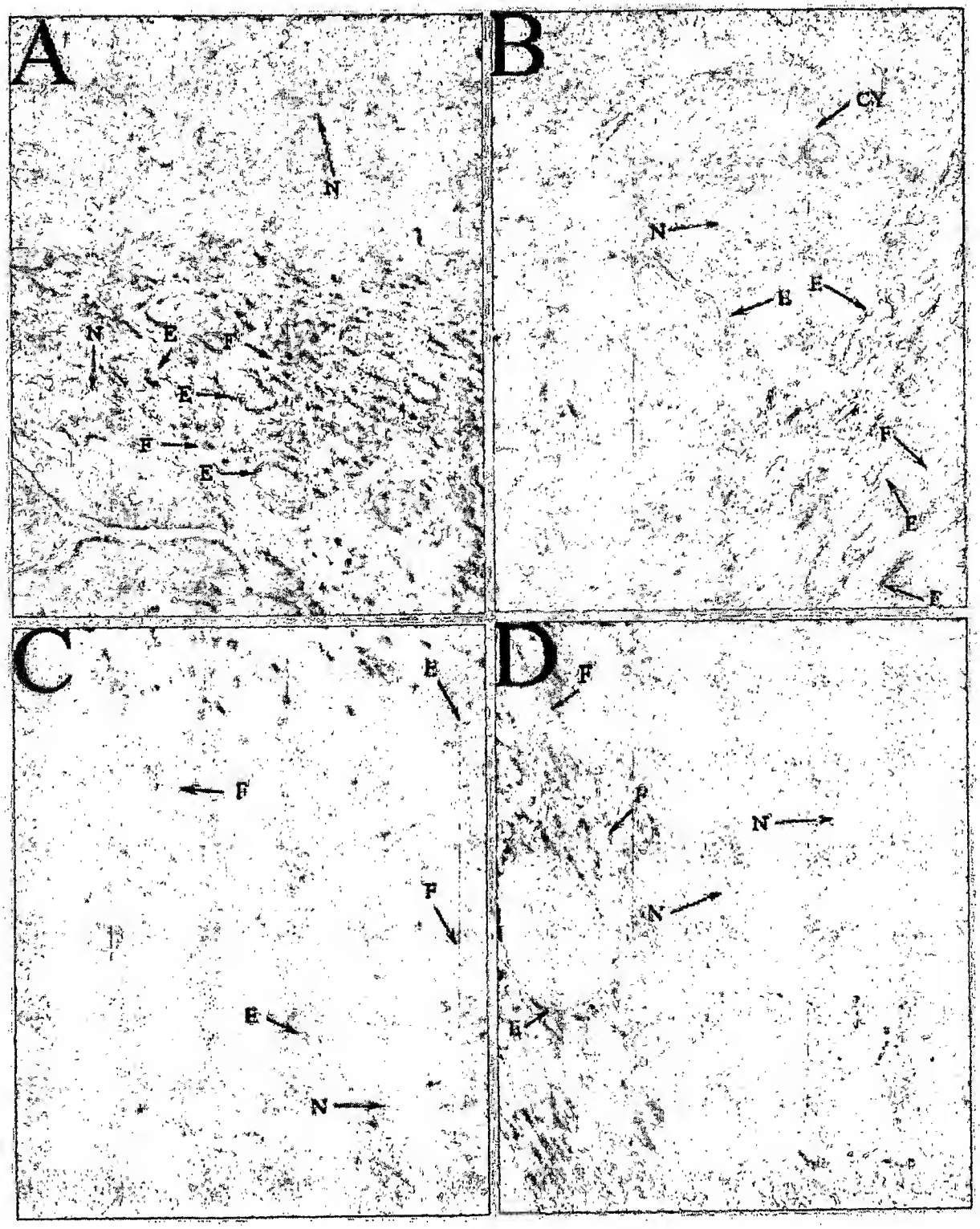

Fig 1

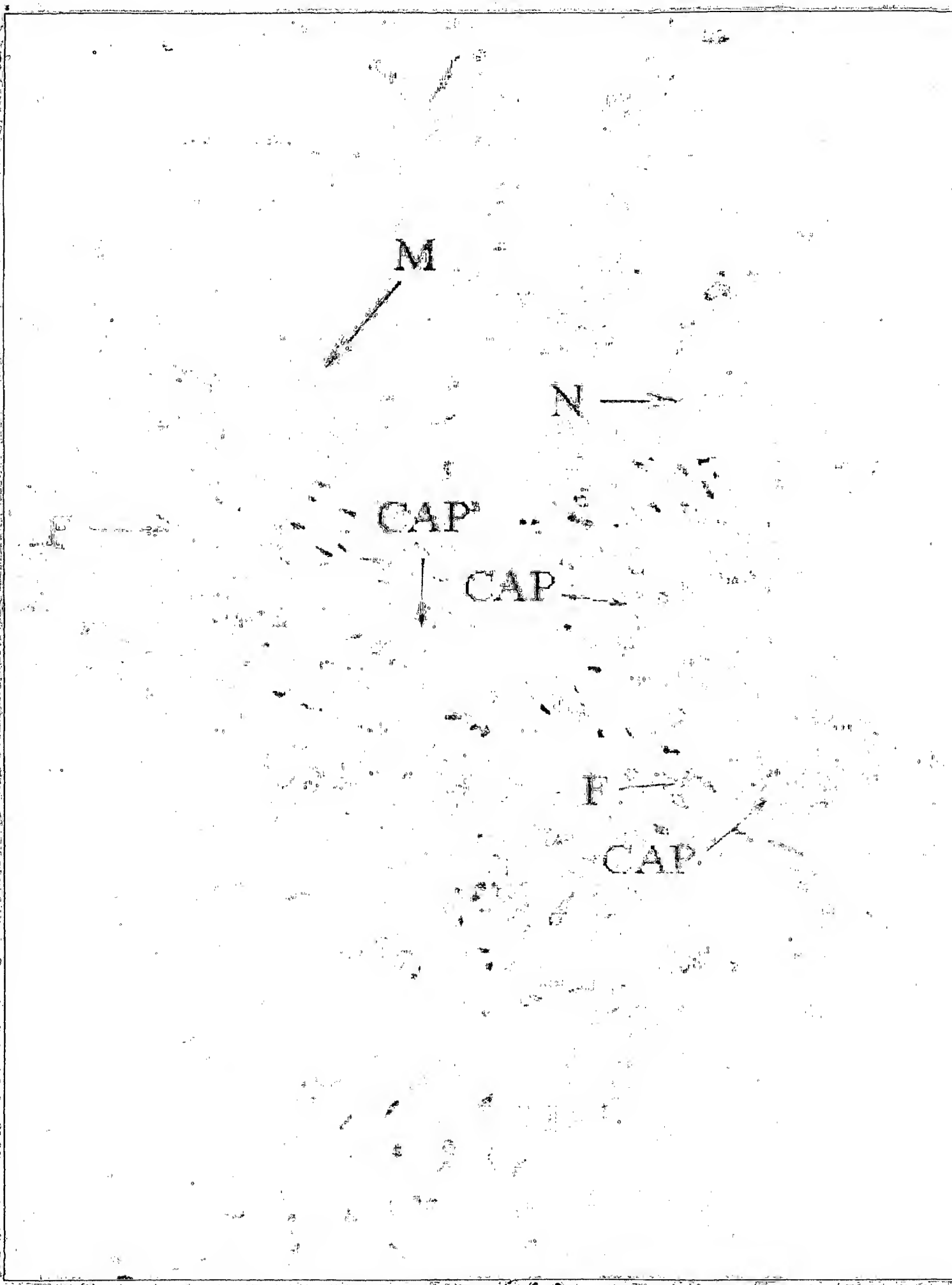

Fig 2

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